Third Edition

Biochemistry

for Medical Laboratory Technology Students

Harbans Lal
Ashuma Sachdeva

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eISBN: 978-93-546-6766-4

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Third eBook Edition: 2024

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Published by Satish Kumar Jain and produced by Varun Jain for
CBS Publishers & Distributors Pvt. Ltd.

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Preface to the Third Edition

As we present the third edition of our *Biochemistry for Medical Laboratory Technology Students*, we reflect on the dynamic landscape of scientific discovery that has unfolded since the inception of the first edition. The field of biochemistry continues to evolve, driven by relentless research and technological advancements.

Building upon the foundation of the previous editions, we have integrated cuting-edge research, refined explanations, and incorporated feedback from educators and students alike. Our aim remains steadfast to demystify the complexities of biochemistry and present it in a very lucid manner so as to be comprehensible and retainable by the MLT students.

From conceptual summaries to thought-provoking questions, we aspire to engage students in active learning and critical thinking. As educators, we are acutely aware of the challenges students face in navigating this intricate field. The third edition is designed to be a reliable companion, offering clarity without sacrificing depth. We hope this textbook serves as a catalyst for intellectual curiosity, inspiring the next generation of students to explore the marvels of biochemistry with enthusiasm and curiosity.

We express our gratitude to the academic community for their continued support and constructive feedback. We also thank Mr SK Jain and his entire team from CBS Publishers & Distributors, for being as passionate about this project. As we embark on this journey with the third edition, we remain commited to fostering a love for learning and empowering students to unravel the mysteries of the biochemical realm.

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Preface to the First Edition

In the era of evidence-based medicine, understanding the subject of biochemistry is very important, yet a difficult proposition for the students, particularly, who are pursuing the course of Medical Laboratory Technology (MLT). While lots of details and up-to-date information is available in various books and on internet, our aim is to present the desired information in a very lucid, methodical and compact way, with suitable illustrations and tables. The purpose is to help the students, digest and assimilate actual biochemical phenomena as they occur in human body and to understand the alterations in diseases. With this aim in mind, we are happy to present *Biochemistry for Medical Laboratory Technology Students*, wherein the whole syllabus has been divided into three Sections. Section 1 includes 'Basic Principles of Biochemistry'; Section 2 contains 'Analytical Biochemistry and Metabolism' while Section 3 describes 'Clinical Biochemistry'.

In this effort of ours, several people have helped us and we are thankful to each one of them. Especially to mention are Dr. Ekta Malik, Professor, Biochemistry, Lady Hardinge Medical College, New Delhi, who has made a significant contribution in two of the chapters; Dr. Monica, Assistant Professor, and Dr. Deepika, Senior Resident, Department of Biochemistry, PGIMS, Rohtak; and Dr. Asha Kumari, Assistant Professor, Biochemistry, SHKG Medical College, Mewat (Haryana), for their timely help during the course of writing this book. We are also thankful to Dr. Sumit Sachdeva, Professor, RIO, PGIMS, Rohtak, for his constant support. We also express our sincere thanks to several MLT students of Medical College, Agroha, just to name few Meenakshi, Mohit Gulati, Sanjeev Sharma and Pradeep, for their constant support. Special thanks are to our all the family members for their patience and support, as this type of project does steal precious moments from their valuable time too. We are also thankful to the team of CBS Publishers & Distributors, New Delhi, for bringing out this much-needed volume in a relevant and concise manner for the MLT students. Last but not the least, we thank the Almighty who has given us inspiration, strength and motivation to complete this project and bring out the useful volume for the ever-improving students of the health system.

Any work of this magnitude cannot be without its inherent flaws and shortcomings. We take full responsibility of that and encourage our readers to render an honest feedback, so that such shortcomings may be corrected and the book is further improved in its later editions.

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Section 1

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Chapter

1

Introduction to Medical Laboratory Technology

Technology (Greek: *techne* means ‘art, skill, cunning of hands”) is the **collection of techniques, skills, methods and processes used in the production of goods or services**, or in the accomplishment of objectives, such as **scientific investigations**. Technology can be the knowledge of techniques, processes, etc. or it can be embedded in machines, computers, devices and factories, which can be operated by individuals without detailed knowledge of the working of such things.

LABORATORY

A **laboratory** (**lab**) may be referred to a **facility that provides controlled conditions in which scientific or technological researches, experiments and measurements are performed**. It may be a **building, part of a building or other place equipped with scientific equipment for doing scientific tests and/or teaching science, or some area where chemicals or medicines may be produced**.

Laboratories used for scientific research may have many forms due to the differing requirements of specialists, in various fields of science. Whatever the type of the laboratory may be, it is a challenging working environment with the aim of protecting people, assets and the environment, from explosives, and avoiding cross-contamination.

A laboratory should:

- be subjected to strict regulatory requirements;
- ensure data storage without gaps or losses.

MEDICAL LABORATORY

A medical laboratory, also referred to as a **clinical laboratory**, is a laboratory **where tests are done on clinical specimen, in order to get information about the health of a patient**, as pertaining to:

- Diagnosis,
- Confirmation of clinical interpretation,
- Monitoring any therapy,
- Establish prognosis,
- Treatment, and
- Screening of the disease.

CLINICAL LABORATORIES SET UP IN A HEALTH INSTITUTION

Types of clinical laboratories in a health institution vary from one place to another. Most commonly included are:

1. Clinical Chemistry Lab

Clinical chemistry (**clinical biochemistry lab**), also referred as chemical pathology laboratory, **usually receives blood, serum** or **plasma**, and **chemically analyzes** it or other body fluids for a variety of substances, such as sugar, lipids, enzymes or hormones, etc.

It may also include **urine analysis** for the measurement of urinary constituents; analysis of body fluids, like ascitic fluid, pleural fluid or CSF for various biochemical components; analysis of calculi from various tissues for their biochemical composition; and blood gas analysis.

Various sections under clinical chemistry include **analysis of blood** or **its components, enzymology, toxicology, endocrinology**, etc.

2. Clinical Microbiology Lab

Clinical microbiology laboratory receives any clinical specimen, including **swab, feces, urine, blood, sputum, cerebrospinal fluid, synovial fluid** or **infected tissue**.

- The work mainly concerned with **culture** is to look **for suspected pathogens**.
- To determine whether the pathogen is sensitive or resistant to a suggested medicine, **sensitivity testing**

is carried out. Thereafter, results are reported with the identified organism(s) and the type of drug(s) that should be prescribed for the patient.

- Routine immunological tests like ELISA for HIV, HBV, HCV, etc.

Clinical microbiology has different units including **bacteriology**, **virology**, **parasitology**, **immunology**, **mycology**, etc.

3. Pathology Lab

Pathology mainly includes **histopathology**, **cytology**, **hematology** and **serology**.

a. **Histopathology processes biopsies** (solid tissues removed from the body) for evaluation at the microscopic level.
b. **In cytopathology**, **smears of cells** from all over the body, such as cervix, are examined for the evidence of inflammation, cancer or any other condition.
c. **Hematology** works with whole blood, to do full **blood counts** and blood films as well as many other specialized immunohematological tests. It also includes **coagulation studies**, such as clotting time and the study of coagulation factors.
d. **Serology** includes the concept of antigen–antibody interaction as a diagnostic tool. It is also used for compatibility of the transplanted organ.

4. Blood Bank

- This section **determines blood groups** and performs **compatibility testing** on blood donors and recipients.
- Prepares **blood components**, derivatives and products for transfusion.
- Determines patient's blood type and **Rh status**.

MEDICAL LABORATORY TECHNOLOGY

Medical laboratory technology, also called **clinical laboratory science**, is an **allied health profession**, which is concerned with the **diagnosis, treatment and prevention of diseases** through the **use of clinical laboratory tests**. The tests help the physicians to detect, diagnose and treat a disease.

MEDICAL LABORATORY TECHNOLOGIST

Medical laboratory technologist (also referred to as **medical laboratory scientist**, **clinical laboratory scientist** or **clinical laboratory technologist**) is a healthcare professional, who performs various laboratory tests, by analysing body fluids and tissues, blood typing, microorganism screening, chemical analysis, cell counts of human body, etc. Thus, they play an important role in collecting the samples, and testing, reporting, quality control and documentation of the various laboratory investigations.

They may work in any area of the medical laboratory, e.g. blood bank, clinical chemistry (chemical analysis of body fluids), hematology, immunology (study of immune system), microbiology (study of bacteria and other disease organisms), etc. They may also work in the areas of cytotechnology (study of human tissue), phlebotomy (collection of blood sample), urine analysis, coagulation studies, parasitology or serology.

Role of Medical Laboratory Technologist

As we know, this is the era of evidence-based medicine (EBM), nearly 60 to 70% of all decisions regarding patient's diagnosis and treatment, and hospital admission and discharge, are based on laboratory test results, thus, medical laboratory technologists play an important role in finding out the cause of the disease, by providing the physician with the required laboratory test results and help the patient to get well soon.

Role of a medical laboratory technologist includes:

1. **Setting up of laboratory equipment:** Usually, their day begins with the setting up of and adjusting the laboratory equipment, including its cleaning, maintenance, sterilization, etc.
2. **Collection of patient's sample:** Throughout the day, their task includes sample collection, and receiving the blood, tissue, urine and other body fluid samples from patients, for which they should ensure proper collection methods and maintain a proper record of collection.
3. **Preparation of the sample:** They are required to handle the specimen carefully and to prepare the sample for all types of tests, and examine them for various chemical constituents (analytes), cells or bacteria. They may also be required **to prepare tissues for microscopic examination** by the pathologist.
4. **Analysis:** They are required to analyze blood sample accurately, for its chemical constituents, perform blood counts and cross match blood samples for transfusion. For the purpose, they should work with appropriate safety precautions. Urgent samples, referred to as STAT, should be given preference.
5. **Reporting of the test results:** It is necessary to keep all reports ready in time, keeping in mind the turnaround time (TAT) of the laboratory.

6. **Quality control:** They are also required to perform equipment validation and calibration, and follow quality control procedures, which help to control different types of errors, viz. preanalytical, analytical and postanalytical errors, in the clinical laboratory.
7. **Supplies:** They are also supposed to keep the reagent bottles, pipettes and other laboratory requirements at the appropriate place after use.
8. **Standardization: Standardize and modify procedures** to ensure accuracy of the test.
9. **Research: Help scientists in research.**

Ethics

Medical laboratory technologists perform various laboratory tests that are crucial for accurate diagnosis and treatment. Thus, maintaining a strong work ethics is necessary to assure the patient and the treating physician that the test results are accurate and reliable. Thus, a medical laboratory technologist should be:

1. **Respectful:** Medical laboratory technologist should value and protect welfare and dignity of all the individuals. They should be respectful, accessible and cooperative with the patients, colleagues and other healthcare professionals, to provide effective patient care.
2. **Maintain patient's dignity:** They must respect dignity and independence of the patient, at all times. Race, religion, occupation, gender and lifestyle must never, negatively, impact professional conduct.
3. **Patient's privacy:** Another important aspect of the ethical behavior is protecting patient's privacy. Since they have access to the sensitive information, they must preserve patient's confidentiality, which otherwise could potentially damage the patient's personal and professional life.
4. **Maintain state-of-the-art skill:** Since technology continuously creates new laboratory testing protocols and equipments, the technologist must accept the fact that lifelong learning is concomitant for pursuing the profession.
5. **Recognise competence of others:** They must recognise competence of others and seek their assistance, as and when required. They should also collaborate with other health professionals in the care of the patient, and functioning and improvement of health services.
6. **Share knowledge:** They must share their knowledge with the colleagues, students and other healthcare providers.
7. **Follow safety procedures:** They should promote a culture of safety. They must compliance with current regulations for protection of patients, colleagues, healthcare providers, society, environment and themselves with the intention of minimizing the risk.

Responsibilities

1. Every medical laboratory technologist must maintain **high moral and professional standard** of behavior. Truth must always be told, because a wrong result may lead to patient's death. The laboratory worker **should not offer personal excuses** for shortcomings in the performance of duty. An error should promptly be reported to the superior without fear, so that it can be corrected timely.
2. It is also necessary to **keep all the reports ready in time**, particularly, the urgent ones.
3. In the course of laboratory testing, laboratory technologist gains information about patient and the illness. They must keep this information **strictly confidential**. Only the physician, who requested the examination, should receive the **patient's report**.
4. The laboratory technologist should **look after all the equipment** carefully and use correct amount of the reagent or the sample, needed for each test.
5. It is necessary to keep the kits, reagent bottles, glassware and other **laboratory requirements at the appropriate place**, after use.
6. At the end of the day, they must keep the laboratory and all equipment, in preparation for the next day.
7. They must perform equipment's **validation** and **calibration**, maintain **quality control**, STAT or run-by-run assessment, statistical control of the observed data and record normal operations.
8. Some **samples may need to be stored** under controlled conditions for future use, accordingly, these samples should be stored with caution.
9. While working with infectious patients and samples, or hazardous chemicals, **safety precautions** must be taken, to avoid infection or injury.
10. The workplace must be clean, sanitized and well lit.
11. They must wear protective clothing, including lab coats, masks, goggles and gloves, particularly, when handling infected specimen.
12. When working in shifts, they must be ready for call in case of emergency.

Important Questions

1. Discuss role of a medical laboratory technologist.
2. Write notes on:
   - i. Medical laboratory technologist
   - ii. Responsibilities of a medical laboratory technologist

Chapter
2

Carbohydrates

Carbohydrates, also called **saccharides** (Greek: *Saccharon* means sugar), are the most abundant biological molecules containing C, H and O, which are combined in the ratio of 1 : 2 : 1, i.e. as $(CH_2O)_n$, where n is 3 or more. They may be defined as the **aldehyde or ketone derivatives of higher polyhydroxyalcohols or their anhydrides**.

FUNCTIONS OF CARBOHYDRATES

1. Monosaccharides are the major sources of fuel for metabolism, being used both as an energy source (glucose being the most important in nature) as well as in biosynthesis of several compounds.
2. When monosaccharides are not immediately needed by many cells they are often converted to more space efficient forms, often polysaccharides. In many animals, including humans, this storage form is glycogen, especially in liver and muscle cells. In plants, starch is used for the same purpose. The most abundant carbohydrate, cellulose, is a structural component of the cell wall of plants and many forms of algae.
3. Excess of the carbohydrates also get converted into fat.
4. Ribose is a component of RNA while deoxyribose is a component of DNA. Lyxose is a component of lyxoflavin found in the human heart.
5. Galactose, a component of milk sugar lactose, is found in galactolipids in plant cell membranes, and in glycoproteins in many tissues.
6. Mannose occurs in human metabolism, especially in the glycosylation of certain proteins.
7. Fructose, or fruit sugar, is found in many plants, and in humans, it is metabolized in the liver, absorbed directly into the intestine during digestion and is found in semen.

CLASSIFICATION OF CARBOHYDRATES

Depending upon the number of the monomeric units present in the molecule, carbohydrates are classified, as shown in Fig. 2.1.

1. Monosaccharides,
2. Disaccharides,
3. Oligosaccharides, and
4. Polysaccharides.

MONOSACCHARIDES

Simple sugars are called monosaccharides. They are the aldehyde/ketone derivatives of the straight chain polyhydroxyalcohols containing 3 or more carbon atoms. Monosaccharides join together in various ways, to form disaccharides, oligosaccharides and polysaccharides.

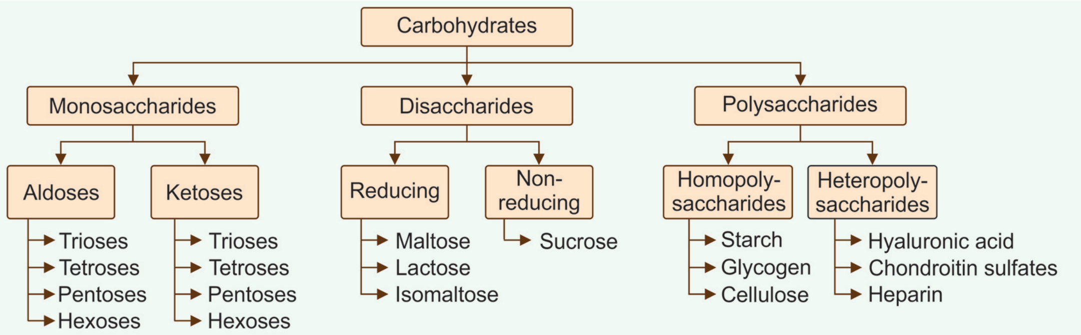

Carbohydrates
Monosaccharides
Disaccharides
Polysaccharides
Aldoses
Ketoses
Reducing
Non-reducing
Homopoly-saccharides
Heteropoly-saccharides
Trioses
Tetroses
Pentoses
Hexoses
Trioses
Tetroses
Pentoses
Hexoses
Maltose
Lactose
Isomaltose
Sucrose
Starch
Glycogen
Cellulose
Hyaluronic acid
Chondroitin sulfates
Heparin

Fig. 2.1: Classification of carbohydrates

Monosaccharides are further classified into different groups (Table 2.1), according to the:

- Chemical nature of the carbonyl group, and
- Number of carbon atoms in the chain.

Table 2.1 Classification of monosaccharides

Mono-saccharides	Number of carbons	Aldoses	Ketoses
Trioses	3	Glyceraldehyde	Dihydroxy-acetone
Tetroses	4	Erythrose	Erythrulose
Pentoses	5	Ribose	Ribulose
Hexoses	6	Glucose	Fructose

According to the Chemical Nature of the Carbonyl Group

According to the chemical nature of the carbonyl group monosaccharides are classified into two groups, as aldoses and ketoses.

Aldoses

A sugar is an aldose when it contains an aldehyde (–CHO) group on C1, e.g. glucose.

Ketoses

The sugar is a ketose when it has a ketone (–CO) group at C2, e.g. fructose. Ketoses are named by inserting '-ul-' before the suffix '-ose', in the name of the corresponding aldose, e.g. erythrulose (ketose of erythrose, which is an aldose).

According to the Number of Carbon Atoms

According to the number of carbon atoms, monosaccharides are classified as trioses, tetroses, pentoses, hexoses, etc.

Trioses

These are the smallest monosaccharides having three carbon atoms, e.g. glyceraldehyde (an aldotriose) and dihydroxyacetone (a ketotriose).

Both the molecules have physiological significance since phosphate esters of these sugars, i.e. glyceraldehyde-3-phosphate and dihydroxyacetone phosphate are important intermediates in glycolysis.

Tetroses

These are the compounds having four carbon atoms, e.g. erythrose (an aldotetrose) and erythrulose (a ketotetrose).

Erythrose, as erythrose-4-phosphate, is found as an intermediate in hexose monophosphate (HMP) shunt.

Pentoses

These are the sugars containing five carbon atoms, e.g. ribose (an aldopentose) and xylulose (a ketopentose). Both of these are found as intermediates in HMP shunt. Ribose also occurs as a constituent of nucleotides found in RNA.

Hexoses

They contain six carbon atoms, e.g. glucose (an aldohexose) and fructose (a ketohexose). Glucose, commonly known as dextrose, is used as a source of energy in the body. Fructose is found as a constituent of the common sugar (sucrose).

Some of the monosaccharides of biological significance are shown in Fig. 2.2.

DISACCHARIDES

Disaccharides are the sugars which consist of two similar or dissimilar units of monosaccharides, which

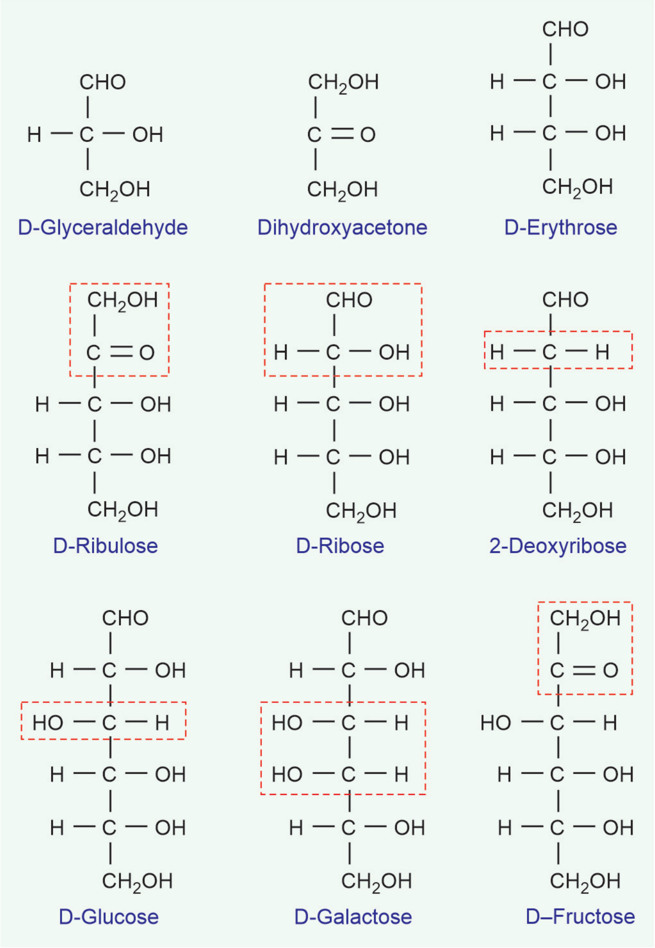


Fig. 2.2: Some monosaccharides of biological importance

are linked to each other by a glycosidic bond, e.g. maltose, lactose and sucrose.

Maltose

Maltose consists of **two glucose** (α-D-glucopyranose) units, which are linked together by **α-1,4-glycosidic linkage**.

- In maltose, C1 of a glucose molecule is linked to C4 of the other molecule of glucose.
- Since –OH group at C4 of a glucose molecule is linked to the –OH group at C1 (the anomeric carbon) of the other glucose molecule, there remains the free –OH group at the anomeric carbon (C1) of the first glucose molecule. Thus, maltose is a **reducing** disaccharide (Fig. 2.3).
- Maltose is obtained as a hydrolyzed product of polysaccharides, such as starch and glycogen.
- Maltose is hydrolyzed to two glucose units by the enzyme maltase, in the intestinal lumen.

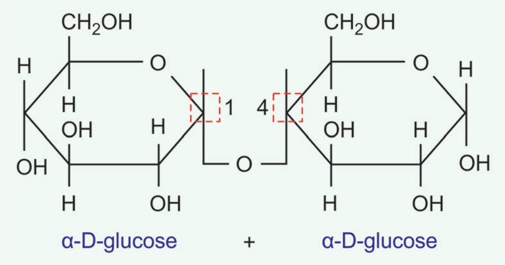


Fig. 2.3: Maltose

Lactose

Lactose consists of **galactose** (β-D-galactopyranose) and **glucose** (β-D-glucopyranose), which are linked together by **β-1,4-glycosidic linkage**.

- In lactose, glycoside bond links C1 of galactose to C4 of glucose.
- There is a free –OH group at the anomeric carbon (C1) of β-D-glucose. Thus, lactose is a **reducing** disaccharide (Fig. 2.4).
- Lactose occurs naturally in milk; hence lactose is also called **milk sugar**.
- In females, lactose is synthesized during lactation and is secreted in milk.
- During late pregnancy and lactation, lactose may be excreted in urine. This is referred to as physiological lactosuria.
- Lactose is hydrolyzed by the intestinal enzyme, called lactase (β-D-galactosidase) to glucose and galactose.

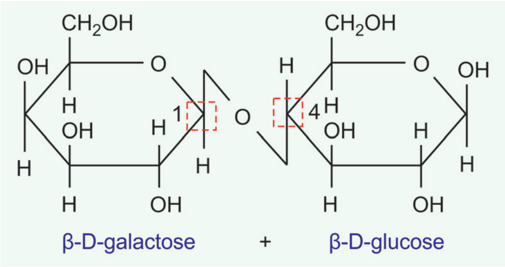


Fig. 2.4: Lactose

Sucrose

Sucrose consists of **glucose** (α-D-glucopyranose) and **fructose** (β-D-fructofuranose), which are linked by the **β-1,2-glycosidic linkage** (Fig. 2.5).

- As carbonyl groups of both the monosaccharides, i.e. C1 of glucose and C2 of fructose, participate in the synthesis of the glycosidic bond and there is no free carbonyl carbon on any of its monosaccharides, thus, sucrose is a **nonreducing** sugar.
- Sucrose is also known as **table sugar**, **cane sugar** or **invert sugar**.
- It is the most abundant disaccharide found in plants.
- Sucrose is hydrolyzed to glucose and fructose by the intestinal enzyme, called sucrase, also referred to as invertase.

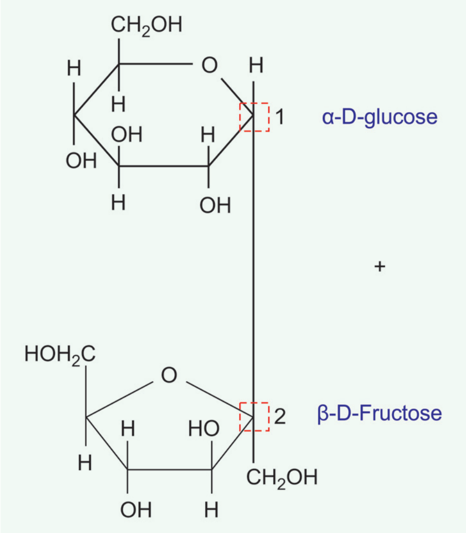


Fig. 2.5: Sucrose

OLIGOSACCHARIDES

Oligosaccharides are the carbohydrates built by the linking of more than two (generally three to ten) monosaccharide units by the glycosidic bonds, e.g. maltotriose, raffinose, etc.

- Oligosaccharides are usually attached to proteins at the sequences that form surface loops or turns.
- A single protein may contain several N- as well as O-linked oligosaccharide chains. Glycosylation may affect structure, stability or activity of the protein.
- High content of O-linked oligosaccharides occurs in mucin.
- Some oligosaccharides also play a structural role.
- Most of these are not digested in human beings.

POLYSACCHARIDES

Polysaccharides, also called **glycans**, are the polymers of large number of monosaccharides, which are linked together by glycosidic bonds.

- Polysaccharides are sparingly soluble in cold water but they form a colloidal solution in hot water.
- They are neither sweet in taste nor have any reducing properties.
- Polysaccharides exist as linear and/or branched polymers.

Polysaccharides are classified into two groups, as homopolysaccharides and heteropolysaccharides.

HOMOPOLYSACCHARIDES

Homopolysaccharides, also called homoglycans, are polymers, which have **identical monosaccharide units**. Homopolysaccharides of biological significance include:

- Homopolymers of glucose, i.e. **starch**, **glycogen** and **cellulose**, and
- Homopolymer of fructose, called **inulin**.

Starch

Starch is stored as a reservoir of food, commonly found in cereals and tubers of plants. Starch consists of two polysaccharide subunits, referred to as α-amylose and amylopectin.

α-Amylose

It is a linear polymer of glucose. Glucose molecules are linked together by **α-(1→4) glycosidic bonds**.

Amylopectin

It is a branched molecule having, both **α-(1→4)** (**straight chain**) and **α-(1→6)** (**branch points**). Branching occurs after every 24 to 30 glucose residues (Fig. 2.6).

- Starch is a main source of carbohydrate in the **human diet**.
- **Amylase**, found in saliva and pancreatic juice, randomly hydrolyses **α-(1→4) glycosidic bonds** of amylose (starch) and degrades starch to give a mixture of maltose, maltotriose and dextrins.

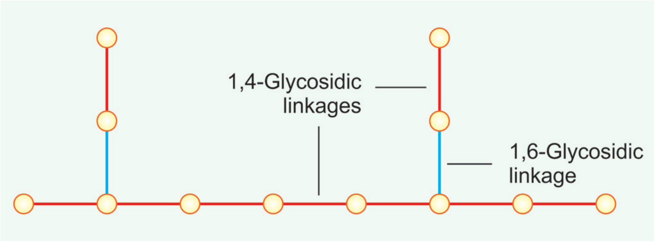


Fig. 2.6: Starch

Glycogen

Glycogen is a polysaccharide found in animals, hence it is also called **animal starch**.

- Although, glycogen is found in all the cells in animals, it is most prevalent in **skeletal muscle** and **liver**.
- In the liver, glycogen is degraded by the enzyme phosphorylase and serves as a readily available source of glucose, especially, during initial 18–24 hours of fasting.
- Structure of glycogen is similar to amylopectin having both, a linear chain consisting of **α-(1→4) glycosidic bonds** and the branch points consisting of **α-(1→6) glycosidic bonds**. However, compared to amylopectin, in glycogen, branching occurs more frequently, i.e. after every 10–12 glucose residues. Thus, glycogen is highly branched, having a tree-like structure (Fig. 2.7).

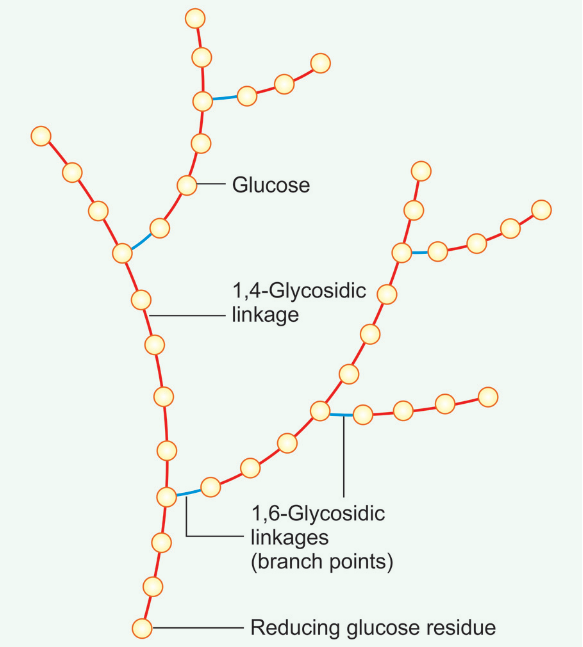


Fig. 2.7: Glycogen

Differences between starch and glycogen are shown in Table 2.2.

Table 2.2 Differences between starch and glycogen

Starch	Glycogen
It is a plant reserve food, mainly stored in grains	It is an animal reserve food, mainly stored in the liver and muscle
It is less branched. Branching occurs after every 24 to 30 glucose units	It is highly branched. Branching occurs after every 10 to 12 glucose units
It consists of two subunits, called amylose and amylopectin	No such structural subunits are found
It is sparingly soluble in cold water and forms a paste in hot water	It forms an opalescent solution in water
It gives violet color with iodine	It gives brown to red color with iodine

Cellulose

Cellulose is found in plants.

- Cellulose is a linear polymer of β-D-glucose, and, thus, has **β-(1→4) glycosidic bonds**.
- It cannot be digested by human beings because they lack the enzyme cellulase, which hydrolyzes β-(1→4) glycosidic linkages, present in cellulose.
- Though cellulose cannot be utilized by human beings, it forms a considerable part of the vegetarian food and adds to the bulk of the feces.

Inulin

Inulin is found in plants, in the bulbs of dahlia, onion and garlic.

- It is not utilized by human beings.
- However, it is of biological significance in humans as it is used for determining glomerular filtration rate **(GFR)**.

Dextrans

Dextrans are produced by various microorganisms, grown in sucrose media and have high molecular weight.

- Dextrans are highly branched homopolymers of **glucose** having **1-6**, **1-4** and **1-3 linkages**.
- They are used for intravenous infusion as plasma volume expanders, in the treatment of hypovolemic shock.
- Dextrans are also used as food additives and cryopreservatives.

HETEROPOLYSACCHARIDES

Heteropolysaccharides (heteroglycans) are the polymers of nonidentical monosaccharide units, also referred to as **glycosaminoglycans** (**GAGs**). Glycosaminoglycans, originally designated as **mucopolysaccharides**, are large complexes of negatively charged heteropolysaccharides associated with a small amount of protein, forming **proteoglycans**. These are the **gel-forming components** of the extracellular matrices. According to their monomeric composition, type of glycosidic linkage, and degree and location of sulfate units, GAGs are divided into six major classes.

Hyaluronic Acid

Hyaluronic acid is composed of repeating disaccharide units of **D-glucuronic acid** and **N-acetyl-D-glucosamine**.

- This polysaccharide chain is the **longest** of the glycosaminoglycans (GAGs).
- It is the only GAG which is **nonsulfated**.
- It is **not covalently attached to protein** and is not limited to animal tissues but is also found in bacteria.
- It is an important component of the **connective tissue**, **synovial fluid** and **vitreous humor in the eye**.
- Viscoelastic behavior of hyaluronate solution makes it an excellent **biological shock absorber** and **lubricant**.
- Its concentration is **decreased in osteoarthritis**.

Chondroitin Sulfates

Chondroitin sulfates contain **glucuronic acid** and **N-acetylgalactose** with sulfate on the hydroxyl group of either carbon-4 (chondroitin-4-sulfate) or carbon-6 (chondroitin-6-sulfate).

- They are the major components of the **cartilage** where they bind to collagen and hold fibers in a tight and strong network.
- They are also found in the **aorta**, **tendons** and **ligaments**.

Dermatan Sulfate

Dermatan sulfate is derived from chondroitin, by enzymatic epimerization of carbon-5 of its glucuronate residues to iduronate residues.

- Dermatan sulfate contains **L-iduronic acid** (with variable amounts of glucuronic acid) and **N-acetylgalactosamine**.
- It is found in **skin**, **blood vessels**, **tendon** and **heart valves**.

Heparan Sulfate

Heparan sulfates consist of **glucuronic** or **iduronic acid**, and **glucosamine**. Some glucosamines are acetylated and may also have N- or O-sulfate groups.

- It is a ubiquitous cell-surface component as well as an **extracellular substance present in blood vessel walls** and **brain**.
- Some heparan sulfates, which function directly at the cell surface, **interact with a variety of proteins**, including some growth factors and growth-factor receptors (such as fibroblast growth factors and their receptors).
- Formation of the ternary complexes of heparan sulfate with fibroblast growth factor and fibroblast growth-factor receptor **initiate signaling processes**.

Heparin

Heparin consists of **glucuronic** or **iduronic acid** and **glucosamine**. Most of its glucosamine units are N-sulfated. Sulfate is also bound to the hydroxyl group on carbon-2 of uronic acid residues and carbon-3 or carbon-6 of glucosamine residues.

- It is a highly charged polymer found in the **intracellular granules of mast cells** that occur **in arterial walls**, especially in liver, lungs and skin.
- Heparin is widely used, clinically, to **inhibit blood clotting**, e.g. in postsurgical patients.
- Suitably modified low-molecular weight heparin is used in the prophylaxis and treatment of deep vein thrombosis.

Keratan Sulfates

Keratan sulfate is the most heterogeneous group of glycosaminoglycans, as its sulfate content is variable. It also contains small amounts of **fucose**, **mannose**, **N-acetylglucosamine** and **sialic acid**. The repeating disaccharide, generally, is N-acetylglucosamine and galactose. Sulfate content is variable and may be present on hydroxyl group at carbon-6.

- Keratan sulfates are **linked to proteins** either by N-linked (keratan sulfate I) or O-linked (keratan sulfate II) oligosaccharides.
- Keratan sulfate I is found in **cornea** while keratan sulfate II occurs in **loose connective tissue** proteoglycan aggregates with chondroitin sulfate.

PROPERTIES OF CARBOHYDRATES

Isomerism

Carbohydrates exhibit different types of isomerism, such as stereoisomerism, optical isomerism, etc.

Stereoisomerism

Carbon-containing compounds commonly exist as **stereoisomers**, the molecules with the same chemical bonds but different stereochemistry, i.e. different configurations (the fixed spatial arrangement of atoms).

Configuration is conferred by the presence of either:

- **Double bond**, around which there is no freedom of rotation, or
- **Chiral centres**, around which substituent groups are arranged in a specific sequence.

Asymmetric Carbon Atom

Asymmetric carbon atom refers to the carbon atom having four different atoms or groups attached to it. It is also referred to as asymmetric centre or chiral centre (Fig. 2.8). All the monosaccharides except dihydroxy-acetone, contain one or more asymmetric carbon atoms.

A molecule with only one chiral centre can have two stereoisomers. When two or more **chiral carbons**, suppose **n**, are present, there can be $\mathbf{2^n}$ stereoisomers.

Enantiomers

A compound having one asymmetric carbon atom may occur in two forms, both of which are the non-superimposable mirror images of each other. They are called **enantiomers**. For example, **D-glyceraldehyde** and **L-glyceraldehyde**.

- D- or L-form of a monosaccharide, in turn, is determined with respect to its **spatial relationship with glyceraldehyde**, which is referred to as the **parent compound of carbohydrate family**.
- **In Fischer projection**, glyceraldehyde molecule with an **–OH group around the asymmetric centre** (i.e. the α-carbon atom or the carbon atom adjacent to the carbon atom with the terminal primary alcoholic group) **on the right-hand side**, when –CHO group is at the top, is designated as **D-glyceraldehyde**.
- Its nonsuperimposable mirror image, which has an **–OH group around the asymmetric centre on the left-hand side** is designated as **L-glyceraldehyde** (Fig. 2.8).

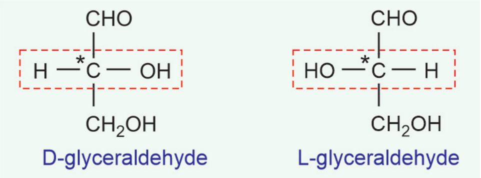


Fig. 2.8: Asymmetric carbon atom (*C)

- Any successive addition of a carbon atom, in the form of –CHOH (secondary alcoholic group), to the parent compound (D-glyceraldehyde), gives rise to the family of **D-sugars**. Thus, D-sugars have same absolute configuration at the asymmetric centre, farthest from their carbonyl group, as does D-glyceraldehyde (e.g. –OH at fifth carbon of D-glucose is on the right hand, similar to the –OH at the second carbon of D-glyceraldehyde).
- Since each new carbon atom (as a secondary alcoholic group) is added as an asymmetric centre, number of isomers increases with the increasing number of carbon atoms. The **number of isomers** so formed, thus, **depends upon the number of the asymmetric carbon atoms**. For example, number of asymmetric carbon atoms in a glucose molecule are four (i.e. n = 4), hence, glucose has **2^4** (16) **stereoisomers**.

Optical Isomerism

Enantiomers have nearly identical chemical properties but differ in a characteristic physical property, i.e. their interaction with the plane-polarized light. In separate solutions, two enantiomers rotate the plane of plane-polarized light in opposite direction.

- An isomer which **rotates the plane of polarized light to the right** is designated as **dextrorotatory** (**d** or **+**).
- The other isomer, which rotates the plane of polarized light to the left is designated as **levorotatory** (**l** or **–**).
- A chiral molecule with D-configuration can be either dextrorotatory [D (+)] or levorotatory [D (–)] indicating its structural relationship to D- or L-glyceraldehyde but exhibiting different types of optical rotation.
- An equimolar solution of the two enantiomers shows no optical rotation and is called a **racemic mixture** (**dl**-mixture).

Epimers

Sugars that differ in configuration of its –H and –OH groups **around only one of the optically active carbon atom** are called **epimers**. For example:

- **D-Mannose** differs in configuration around the second carbon atom (C2) with respect to D-glucose.
- **D-galactose** has a different configuration than D-glucose around the fourth carbon atom (C4).
- D-Mannose and D-galactose, therefore, are the **epimers of D-glucose** (Fig. 2.9).

Formation of Ring Structure

- Generally, monosaccharides have ring conformation.
- Cyclization of **D-glucose** forms a six-membered ring, which has a hemiacetal bond between the aldehyde carbonyl group of carbon-1 and the hydroxyl group of carbon-5. The ring structure, so formed, is analogous to the structure of **pyran**, which is a six-membered ring that has five carbon atoms and one oxygen atom (Fig. 2.10).

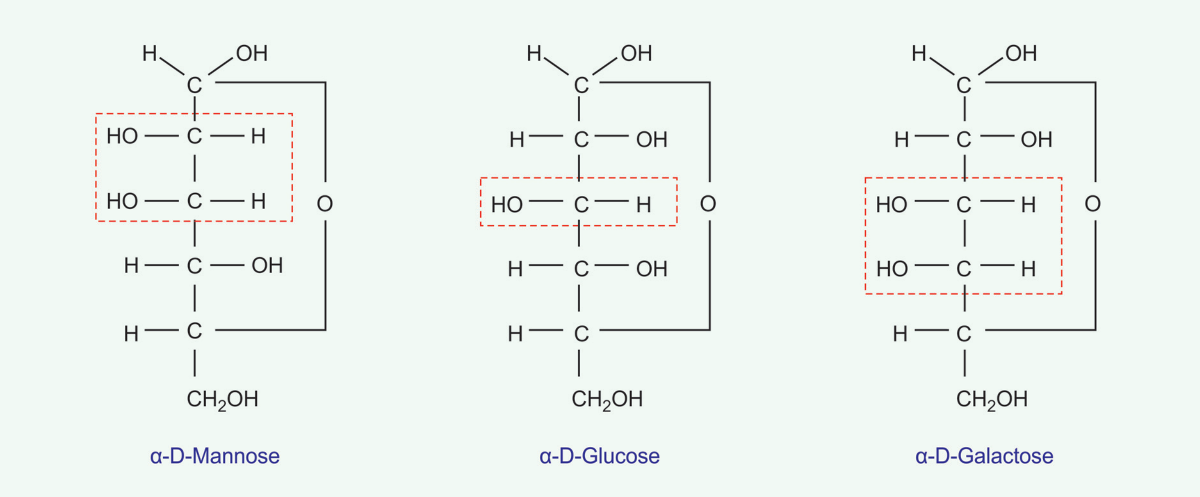


Fig. 2.9: Epimers of glucose

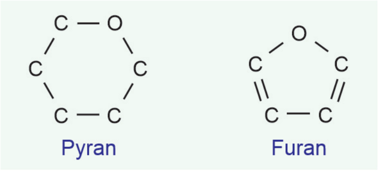


Fig. 2.10: Pyran and furan rings

- Monosaccharides having pyran ring are designated as **pyranoses**. Accordingly, glucose is referred to as **glucopyranose**.
- On the other hand, cyclization of **D-fructose** forms a five-membered ring, which has a hemiketal bond between the carbonyl group of carbon-2 and the alcoholic group of carbon-5. The ring structure, so formed, is analogous to the structure of **furan**, which is a five-membered ring that has four carbon atoms and one oxygen atom (Fig. 2.10).
- Monosaccharides having furan rings are designated as **furanoses**. Accordingly, fructose is referred to as **fructofuranose**.
- The smallest monosaccharide having a furan ring is ribose.
- Ring structures of monosaccharides were proposed by Haworth and, thus, are also called **Haworth** structures.
- When a monosaccharide cyclizes, its **carbonyl carbon**, called **anomeric carbon**, also becomes asymmetric and exhibits two configurations, which are referred to as α and β forms.

Anomers

Isomeric forms of monosaccharides that differ only in their configuration at the anomeric carbon, i.e. the first carbon (C1) in an aldose (e.g. glucopyranose) or the second carbon (C2) in a ketose (e.g. fructofuranose) are called **anomers**.

- An **α-anomer** has **–OH group** (at the anomeric carbon) **below the plane**.
- The other anomer, which has **–OH group** at the anomeric carbon **above the plane** is designated as **β-anomer** (Fig. 2.11).

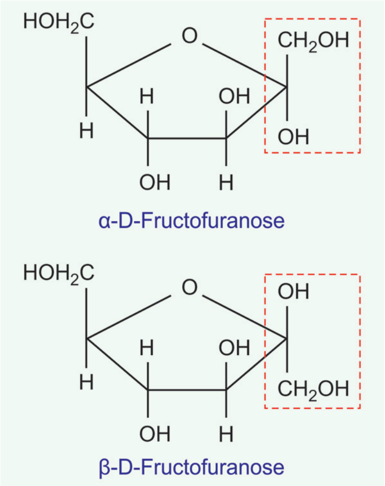


Fig. 2.11: α and β anomers of fructose

- Two anomers of D-glucose, i.e. α-D-glucopyranose and β-D-glucopyranose, have different physical and chemical properties, including optical rotation.
- They are, however, freely interconvertible in an aqueous solution.
- During equilibrium, optical rotation of D-glucose also changes with time and reaches a constant value of 52.5°.
- At equilibrium, D-glucose is a mixture of β-anomer and α-anomer, in a ratio of nearly 2 : 1.

Mutarotation

The phenomenon of **change in optical rotation** of D-(+)-glucose in an aqueous solution and reaching a constant value (due to interconversion of the two anomers) at equilibrium, is called mutarotation (Fig. 2.12).

Reducing Properties

All the monosaccharides and disaccharides (except sucrose) have reducing (**oxidation–reduction**) properties. They act as reducing agents because they have a free aldehyde or ketone group. They reduce some other compound and, in turn, get oxidized themselves.

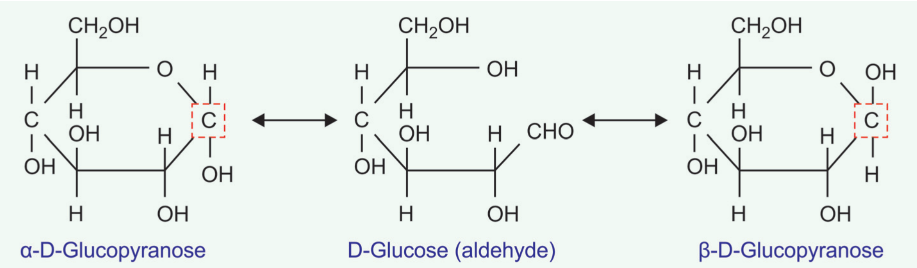


Fig. 2.12: Mutarotation of glucose

Monosaccharides can be oxidized by, relatively, mild oxidizing agents, such as ferric (Fe^{3+}) or cupric (Cu^{2+}) ions. This property forms the basis of the Fehling's reaction, a qualitative test for the presence of reducing sugar.

- **Oxidation of the carbonyl (aldehyde) carbon** of an aldose (C1) to the carboxyl group produces **aldonic acid**, such as D-gluconic acid, which is formed by the oxidation of D-glucose.
- **Oxidation of the carbon at C6** forms **uronic acid**, such as **D-glucuronic acid**, which is formed from glucose (Fig. 2.13).

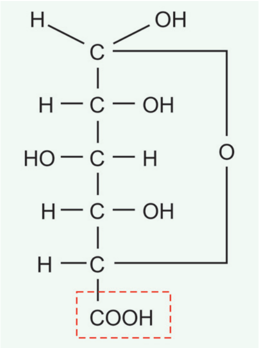


Fig. 2.13: D-Glucuronic acid

- **Oxidation of both the carbon atoms (i.e. C1 as well as C6) forms saccharic or aldaric acid.**
- **Reduction of monosaccharides** under mild conditions produces cyclic polyhydroxy alcohols, called **alditols**. For example, ribitol (from ribose), xylitol (from xylose), sorbitol (from glucose), etc.

Dehydration

A dehydration reaction is usually defined as a chemical reaction that involves the loss of a molecule of water from the reacting molecule.

- Dehydration reactions are a subset of condensation reactions.
- Dehydration reactions and dehydration synthesis have the same meaning, and are often used interchangeably.
- Two monosaccharides, such as glucose and fructose, can be joined together (dehydration synthesis), using sulfuric acid. The new molecule, consisting of two monosaccharides, is called a disaccharide (sucrose).
- Monosaccharides, when treated with concentrate sulfuric acid, undergo dehydration, which, in turn, removes three molecules of water. Based on this reaction, hexoses form hydroxymethylfurfurals while pentoses form furfurals.

Osazone Formation

This is based on the principle that when a solution of a reducing sugar is heated, in a water bath, in an acidic medium with phenylhydrazine, phenylhydrazone (osazone) crystals are formed whose shape vary with the type of the sugar.

- This reaction is used to identify sugar, where glucose gives broomstick or needle-shaped crystals while maltose forms sunflower-shaped crystals.
- In this reaction, first two carbon atoms of the sugar molecule participate and react with 3 molecules of phenylhydrazine, i.e.:

  Hexose + 3 molecules of phenylhydrazine → Osazones
- Three monosaccharides, i.e. glucose, fructose and mannose, give similar types of osazones. This is due to the reason that they differ structurally, only in the first two carbon atoms, which participate in the reaction.

Ester Formation

Esters are the chemical compounds, which are derived from an acid where at least one of the –OH (hydroxyl) group is replaced by the –O– (alkyl) group.

- Usually, esters are formed by the reaction of a carboxylic acid with an alcohol.
- Sugars, by virtue of having alcohol groups, readily reacts with acids and form esters.
- Most important esters of sugars are formed with phosphoric acid, e.g. glucose-6-phosphate, glucose-1-phosphate, pentose-phosphates, etc.

Glycosides

A **glycoside** is a molecule that is formed by the condensation reaction, between a sugar and the hydroxyl group of some other compound, which may be another sugar (glycone) or some nonsugar molecule (aglycone).

- Glycosides can be linked by O- (an O-glycoside), N- (a glycosylamine), S- (a thioglycoside), or C- (a C-glycoside) glycosidic bond.
- If the carbohydrate portion is glucose, the resulting glycoside is a glucoside; if galactose, it is referred to as a galactoside, etc.
- Carbon atom number-1 of a monosaccharide can also react with an alcohol, such as methanol, and form methylglycosides. Methylglycosides are found in glycosaminoglycans.
- Molecules containing N-glycosidic bonds are known as glycosylamines.

COLOR REACTIONS OF CARBOHYDRATES

Due to the presence of a reducing group (aldehyde or ketone group) or the presence of different monosaccharide units, several tests can be performed for the detection of a carbohydrate. The unspecific tests include Molisch's test, which is based on the formation of a furfural, or its derivative, in the presence of concentrated acid. Other tests, e.g. Fehling's test, Benedict's test, etc. are based on their reducing properties. Complex formation, such as formation of the phenylhydrazone crystals is used as a specific test for the identification of some of the carbohydrates, especially disaccharides. For the identification of polysaccharides, iodine is commonly used.

REACTION FOR THE IDENTIFICATION OF A CARBOHYDRATE

Color reaction for the identification of a carbohydrate is called Molisch's test. This test just indicates the presence of a carbohydrate. The reaction is due to the formation of furfural or its derivatives, which are highly reactive. They react with a number of compounds and produce complex-colored products.

For this purpose, take about 2 mL of a carbohydrate solution in a test tube. Add a drop of the Molisch's reagent and mix, cautiously. Continuously pour down about 1 mL of concentrated H_2SO_4 in the way that two distinct layers are formed (the bottom layer being that of the acid). A reddish-violet zone appears at the junction of the two layers, indicating the presence of carbohydrate.

Molisch's reagent is 10% solution of α-naphthol in ethanol.

REACTIONS FOR A REDUCING SUGAR

As discussed above, due to the presence of a free aldehyde or ketone group, carbohydrates are readily oxidized and behave as reducing agents. Therefore, only the reducing sugars will give a positive reaction. Nonreducing sugar, like sucrose, can respond to these reactions after sucrose is hydrolyzed to the monosaccharide units (glucose and fructose).

Tests based on the reducing properties of a sugar include:

Fehling's Test

In this reaction, there occurs the reduction of copper sulfate ($CuSO_4$) to cuprous oxide (Cu_2O), in an alkaline medium.

Add 1 mL of a sugar solution (a few drops at a time) to 4 mL of warm Fehling's solution and heat the mixture. Red precipitate indicates the presence of a reducing sugar.

Benedict's Test

In this reaction also, there occurs the reduction of copper sulfate ($CuSO_4$) to cuprous oxide (Cu_2O), but in the weak-alkaline medium. Benedict's test, however, is more specific for a reducing sugar than the Fehling's test.

Whereas in the Fehling's test, reduction of $CuSO_4$ takes place in a strong-alkaline medium where even a small quantity of a reducing substance gives red precipitate. On the other hand, in the Benedict's test reduction occurs in a weak-alkaline medium. Hence, Fehling's test is more sensitive but less specific while Benedict's test is less sensitive but more specific.

Benedict's test is routinely used in the examination of urine, for the detection of a reducing sugar.

Benedict's qualitative solution is prepared by dissolving sodium citrate and sodium carbonate in water. Thereafter, to the above citrate–carbonate mixture, copper sulfate solution is slowly added with constant stirring. In the presence of a reducing sugar, it thus gives a red precipitate.

Add 5 mL of the Benedict's reagent (qualitative Benedict's reagent) to 0.5 mL of the sugar solution in a test tube. Mix and heat the contents. In case of a reducing sugar, there is the appearance of red precipitate.

Barfoed's Test

Barfoed's test is also a copper reduction test. It, however, differs from the Fehling's test and the Benedict's tests in the way that in the Barfoed's test, reduction of copper sulfate occurs in the acidic medium compared to the alkaline medium in the other two tests.

Barfoed's reagent is prepared by dissolving neutral copper acetate in water. Thereafter, glacial acetic acid is added to it.

Mix 5 mL of the Barfoed's reagent with 0.5 mL of the carbohydrate solution in a test tube and heat in a boiling water bath for 5 min. Appearance of the red precipitate within 5 min indicates the presence of a monosaccharide. If red precipitate appears after 5 min, it confirms the presence of a reducing disaccharide (lactose or maltose).

It is due to the reason that when a sugar solution is boiled with the Barfoed's reagent, a disaccharide is hydrolyzed by the acetic acid (present in the reagent) and the constituent monosaccharides give a positive test. Hence, monosaccharides respond quickly than a disaccharide.

Test for Pentoses

Pentoses react with HCl to form furfural, which, subsequently, reacts with some phenolic compound such as orcinol and forms a deep-green colored complex.

Take 3 mL of the orcinol reagent in a test tube and add 0.5 mL of the sugar solution to it. Boil the contents. Presence of a deep-green color indicates the presence of a pentose.

Orcinol reagent contains orcinol and ferric chloride in concentrated HCl.

Test for Galactose

Galactose forms a red-colored complex with fluoroglucinol in concentrated HCl.

Lactose, after hydrolysis with the hot acid reagent, also gives positive test due to the presence of galactose in it.

Take 2 mL of sugar solution in a test tube. To it, add 2 mL of concentrated HCl and 1 mL of fluoro-glucinol solution. Boil the contents slowly. A red color confirms the presence of galactose. This test is referred to as **Tollen's test.**

Test for Keto Sugars

Presence of a keto sugar such as fructose can be seen by the **Saliwanoff's test**.

The Saliwanoff's reagent contains resorcinol in concentrated HCl.

To 0.5 mL of the sugar solution, add 5 mL of the Saliwanoff's reagent. A blank (containing water) is also prepared (without the sugar solution) to assess the color. Place the test tube in a boiling water bath for 1 min. A ketose (fructose) gives fiery-red color within one minute.

Sometimes, even aldoses, such as glucose, may give weak test, due to the conversion of the small amount of glucose to fructose, if boiling is prolonged. This test may also be given by sucrose, which, during the course of the test, is hydrolyzed to its constituent monosaccharides (glucose and fructose). However, with glucose or sucrose, the test will be positive only after 1 min.

Phenylhydrazine Reaction

As discussed above, when a solution of some reducing sugar is slowly heated in a water bath, in the acidic medium, with phenylhydrazine, it results in the formation of phenylhydrazone (osazone) crystals whose shape vary with the type of the sugar. Whereas, fine needle-shaped yellow crystals are characteristic of a monosaccharide (glucose, mannose or fructose). All these hexoses give identical osazones, due to the reason that except the first two carbons (which participates in the reaction), all the remaining four carbons have same configuration in the three sugars. Sucrose does not form osazone crystals, because of not having a free reducing group available for its reaction with phenylhydrazine.

Take about 200–300 mg (nearly 2–3 spoons) of phenylhydrazine mixture in a test tube. Add few drops of glacial acetic acid and about 5 mL of the sugar solution. Shake well and heat the content in a boiling water bath for 15–30 min. Remove the test tube and allow it to cool, slowly. Yellow crystals of phenylhydrazine, called osazone crystals, appear. Crystals of maltose and lactose appear only on cooling and not in the hot solution while that of glucose appear even when the tube is in the water bath.

Collect some of the crystals by a pipette, transfer them to a glass slide, and examine under microscope. Crystals of lactose are puff-shaped while those of maltose are petal-shaped.

Phenylhydrazine mixture is prepared by mixing equal quantities of the phenylhydrazine hydrochloride with anhydrous sodium acetate.

IODINE REACTION FOR POLYSACCHARIDES

Take 5 mL of starch solution in a test tube. Add a drop of dilute iodine solution and note the presence of blue color. This test is called **iodine test**.

Heat the test tube, blue color disappears but reappears on cooling. Blue colored-substance is an adsorption complex between starch and iodine. Since the iodine–starch complex dissociates on heating, the color subsequently disappears on heating. This complex is formed under neutral or acidic conditions only. This is due to the reason that under alkaline conditions I_2 reacts with NaOH and the blue color disappears. After it is neutralized with acid, I_2 is released from the alkali complex, blue color reappears.

Color given by iodine with the polysaccharide depends upon the chain length of the molecule. Whereas, amylose gives deep blue color; amylopectin (branched chain compound) gives purple color, while glycogen (highly branched structure) gives reddish-brown color. Dextrin formed by the partial degradation of starch gives brownish-red color as the linear chain length is reduced. Extensive degradation of starch may not give any color with iodine.

Important Questions

1. Define carbohydrates. Give their classification. Explain stereoisomerism of carbohydrates.
2. What are polysaccharides? Classify them.
3. Write notes on:
    i. Cellulose
    ii. Anomers
    iii. Epimers
    iv. Mutarotation
    v. Inulin
    vi. Homopolysaccharides
    vii. Heteropolysaccharides
    viii. Hyaluronic acid
    ix. Chondroitin sulfates
    x. Heparin
    xi. Glycosaminoglycans
    xii. Glycosides

Chapter

3

Lipids

Lipid (Greek: *lipos means* fat) is a heterogeneous group of hydrophobic organic molecules found in cells.

- Lipids are not soluble, or are only sparingly soluble, in water but are soluble in organic solvents, such as acetone, ether, alcohol and chloroform.
- Due to this reason, lipids can be easily separated from other biological material after their extraction with some organic solvent.

Physiological Functions of Lipids

1. Due to their high calorific value, fat forms an important dietary constituent of food.
2. Dietary lipids serve as a source of fat-soluble vitamins and essential fatty acids.
3. Fat is the storage form of energy in adipose tissue.
4. Fat presents in the subcutaneous tissues acts as an insulating material.
5. Lipids, in the form of a bilayer together with proteins, form important components of biological membranes.
6. Lipids also act as signalling molecules.
7. Nervous system is very rich in lipids, which are essential for its proper functioning.
8. Lipoproteins are the carriers of triglycerides, cholesterol and phospholipids in the body.

Classification of Lipids

Lipids exhibit great structural variability. They constitute a category of substances that are hydrophobic in nature and are soluble in nonpolar solvents. Lipids are classified into three groups (Fig. 3.1).

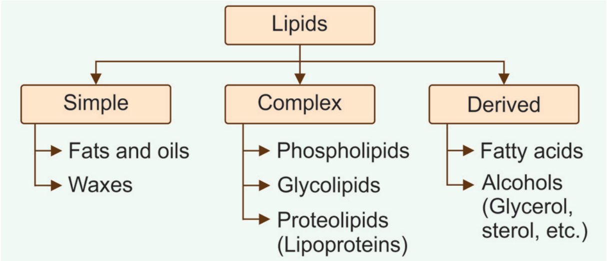


Fig. 3.1: Classification of lipids

Simple Lipids

Simple lipids are the **esters of fatty acids with alcohol**. These include neutral fats, oils and waxes. Neutral fats and oils are the mixtures of triacylglycerols (also called triglycerides) whose fatty acids composition may vary with the source. They differ from each other in the way that fats are solid while oils are liquid at room temperature.

- **Neutral fats** are obtained from animal sources. They have, comparatively, more number of saturated fatty acids and have higher melting points.
- **Oils** are obtained from plant seeds. They are usually rich in unsaturated fatty acids and have low melting points.
- **Waxes** are the esters of long chain fatty acids with high molecular weight mono-hydroxy aliphatic alcohols. They are used in the manufacturing of wax-polish.

Complex Lipids

In addition to fatty acids and alcohol, complex lipids also contain an amphipathic group. For example, lipids conjugated with phosphoric acid are called **phospholipids**, lipids conjugated with sugars are referred to as **glycolipids**, etc.

Derived Lipids

Derived lipids are obtained after the hydrolysis of the simple and compound lipids. They include **fatty acids**, **glycerol**, etc.

FATTY ACIDS

Fatty acids are the carboxylic acids with a long hydrocarbon side chain that may be **saturated** or **unsaturated** (having one or more double bonds).

- Major source of the circulating free fatty acids is adipose tissue.
- After intraplasmic hydrolysis of the triacylglycerols, in chylomicrons and very low density lipoproteins, free fatty acids enter plasma and serve an important source of energy for many tissues.
- Since free fatty acids have limited solubility in water, they are transported in plasma bound to albumin, which has specific binding sites for free fatty acids.

Most of the naturally occurring fatty acids have even number of carbon atoms since they are synthesized from acetyl-CoA, i.e. from two carbon units.

According to their chain length, fatty acids are divided into three groups, referred to as:

- **Short chain fatty acids**, which contain 4 to 8 carbon atoms
- **Medium chain fatty acids**, which contain 10 to 14 carbon atoms, and
- **Long chain fatty acids**, which contain 16 or more carbon atoms.

All types of fatty acids have different physicochemical properties and are metabolized in the body in different ways. In plants and higher animals, predominant fatty acids are those containing 16 and 18 carbon atoms, e.g. palmitic acid, oleic acid, linoleic acid, stearic acid, etc.

SATURATED FATTY ACIDS

Saturated fatty acids have a suffix '**-anoic**' after the name of the hydrocarbon chain, e.g. octadecanoic acid, a saturated fatty acid having 18 carbon atoms.

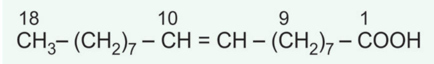
- Saturated fatty acids are highly flexible molecules and can assume a wide range of conformations because there is relatively free rotation around the C–C bond.
- They are commonly found in fats, which are obtained from the animal source, such as butter.
- Saturated fatty acids of common biological significance are listed in Table 3.1.

UNSATURATED FATTY ACIDS

Unsaturated fatty acids have a suffix '**-enoic**' after the name of the hydrocarbon chain, e.g. octadecanoic acid, an unsaturated fatty acid having 18 carbon atoms and one double bond. It is also called oleic acid. Unsaturated fatty acids may have one or more double bonds.

Nomenclature of an Unsaturated Fatty Acid

Widely used convention, to represent an unsaturated fatty acid, is to indicate the **number of carbon atoms**, followed by the **number of double bonds** with **their positions** in the hydrocarbon chain. For example, octadecanoic acid, a fatty acid with 18 carbon atoms and one double bond present after carbon atom number 9 (i.e. between carbon atoms number 9 and 10), is represented as 18:1; 9 (Fig. 3.2).



$$\overset{18}{CH_3}-(CH_2)_7-\overset{10}{CH}=\overset{9}{CH}-(CH_2)_7-\overset{1}{COOH}$$

Fig. 3.2: Oleic acid—an unsaturated fatty acid

An unsaturated fatty acid may be identified either by the delta or the omega system of nomenclature.

Delta System of Nomenclature

According to the delta (δ) system of nomenclature, carbon atoms in the fatty acid chain are numbered from the carboxy-terminal end. This carbon atom (–COOH) is labeled as carbon atom number 1. The carbon atom next to the carboxyl group, i.e. the α-carbon atom, is referred to as carbon atom number 2, and so on.

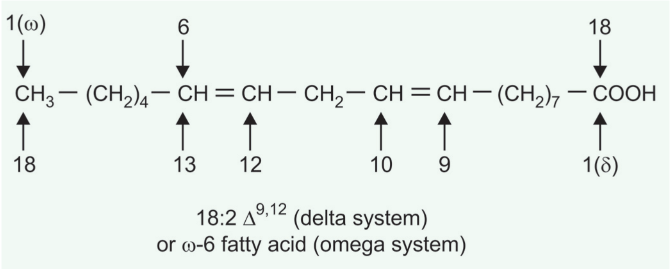
Table 3.1 Saturated fatty acids

Fatty acids	Number of carbons	Formula	Sources
Acetic acid	2	CH_3–COOH	Fermentation of carbohydrates
Butyric acid	4	$CH_3–(CH_2)_2–COOH$	Butter
Caproic acid	6	$CH_3–(CH_2)_4–COOH$	Butter, fat from plant sources
Caprylic acid	8	$CH_3–(CH_2)_6–COOH$	-do-
Capric acid	10	$CH_3–(CH_2)_8–COOH$	-do-
Lauric acid	12	$CH_3–(CH_2)_{10}–COOH$	Coconut oil
Myristic acid	14	$CH_3–(CH_2)_{12}–COOH$	-do-
Palmitic acid	16	$CH_3–(CH_2)_{14}–COOH$	Animal and plant fats
Stearic acid	18	$CH_3–(CH_2)_{16}–COOH$	-do-
Arachidic acid	20	$CH_3–(CH_2)_{18}–COOH$	Peanut oil

Accordingly, an unsaturated fatty acid is represented by:

- A **number**, showing the number of carbon atoms, **followed by** the symbol colon (:) and another number, which represents the number of double bonds.
- **This number is further followed by** the symbol 'Δ' **and some other numbers as superscript**, which indicate **positions of the double bonds**.

For example, linoleic acid, a fatty acid with 18 carbon atoms and two double bonds, which are present after carbon atom numbers 9 and 12, is represented as 18:2 $\Delta^{9,12}$ (Fig. 3.3).



$$CH_3-(CH_2)_4-CH=CH-CH_2-CH=CH-(CH_2)_7-COOH$$

Fig. 3.3: Linoleic acid

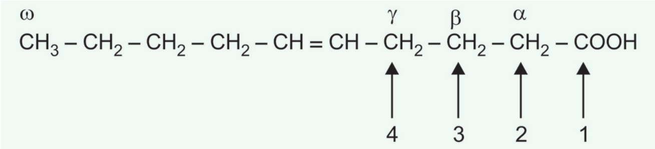
Omega System of Nomenclature

According to the omega (ω) system of nomenclature, carbon atoms are numbered from the methyl end. This carbon is referred to as omega (ω) carbon.

Accordingly, an unsaturated fatty acid is represented by:

- The omega symbol (ω), followed by a number.
- The number followed by the symbol ω represents the number of the carbon atom immediately after which the first double bond appears from the methyl end.

For example, in linoleic acid since the first double bond from the ω-end is present after the sixth carbon atom, the linoleic acid is, thus, referred to as ω-6 fatty acid (Fig. 3.4).



$$\overset{\omega}{CH_3}-CH_2-CH_2-CH_2-CH=CH-\overset{\gamma}{CH_2}-\overset{\beta}{CH_2}-\overset{\alpha}{CH_2}-COOH$$

Fig. 3.4: Omega system of nomenclature of a fatty acid

Polyunsaturated Fatty Acids

Unsaturated fatty acids having two or more double bonds are referred to as polyunsaturated fatty acids (PUFA). They are obtained from plant seed oils, e.g. soybean oil, sunflower oil, groundnut oil, etc. They perform various functions:

1. PUFA form an **important constituent of biological membranes**.
2. They are essentially required for the **esterification of cholesterol**, and, thus, help in its transport and metabolism. Particularly, consumption of fat containing ω-6 fatty acids helps in lowering plasma cholesterol level.
3. Diet rich in ω-3 fatty acids **helps in lowering plasma level of triacylglycerols**.
4. Arachidonic acid also acts as a precursor of some pharmacologically important compounds, such as prostaglandins, thromboxanes and leukotrienes. These are referred to as **eicosanoids**.

Essential Fatty Acids

Linoleic acid, linolenic acid and arachidonic acid are referred to as essential fatty acids since they form an essential component of the diet and are necessary for growth and normal health. They are not synthesised in our body and serve a number of important functions, like:

1. Synthesis of **prostaglandins**.
2. Serve as structural element of **mitochondrial membrane**.
3. Help in the prevention of **fatty liver**.
4. Play an important role in vision.

TRIACYLGLYCEROLS

Triacylglycerols, also called **triglycerides** or **neutral lipids**, are fatty acid tri-esters of glycerol (glycerin, a tri-hydric alcohol).

- Glycerol with one molecule of fatty acid is called **monoacylglycerol**, with two molecules of fatty acids as **diacylglycerol** and with three molecules of fatty acids as **triacylglycerol** (Fig. 3.5).

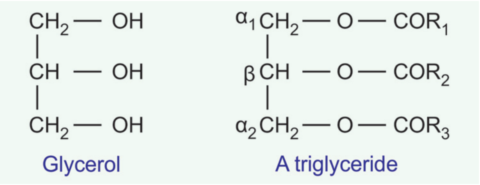


Fig. 3.5: Glycerol and a triacylglycerol

- A molecule of triacylglycerol may have three **similar or dissimilar fatty acids**, which may be **saturated and/or unsaturated**.
- All the natural fats and oils, generally, have three **dissimilar fatty acids**, both **saturated as well as**

unsaturated. These are referred to as **mixed triacylglycerols**.

- Plant oils are usually rich in unsaturated fatty acids.
- Triacylglycerols are transported in plasma, in chylomicrons and very low density lipoproteins (VLDL).

Nomenclature of Triacylglycerols

Triglycerides are named according to the presence of the fatty acids that are attached to glycerol, e.g. a triacylglycerol having palmitic acid at carbon-1, arachidonic acid at carbon-2 and stearic acid at carbon-3 of glycerol is called 1-palmitoyl-2-arachidonoyl-3-stearoylglycerol.

Generally, saturated fatty acids are present at the primary alcoholic groups, i.e. at the α-positions of glycerol (position numbers 1 and 3), whereas an unsaturated fatty acid is present at the β-position (position number 2).

PHOSPHOLIPIDS

Phospholipids are a heterogeneous group of compounds, which contain an alcohol and one or more phosphoric acid molecules.

- A phospholipid has one or two long chain fatty acids that are linked to the alcohol.
- In addition, phosphoric acid may be attached to some polar group, which may be some nitrogenous compound or a polyhydroxy alcohol.
- Thus, phospholipids are **amphipathic** in nature, i.e. they have a **hydrophilic head** and a long **hydrophobic tail**.
  - Its **hydrophilic domain** interacts with the aqueous environment and comprises an alcohol, a phosphate group and some polar compounds, such as serine, ethanolamine, or choline.
  - The **hydrophobic domain** forms non-aqueous environment and contains long chain fatty acid(s) or a fatty acid-derived long hydrocarbon chain.

Physiological Functions of Phospholipids

1. Phospholipids are the predominant lipids of a cell membrane.
2. Membrane phospholipids also function as a reservoir for the intracellular messengers.
3. They also serve as anchors for some proteins in cell membranes.
4. Phospholipids also function as lung surfactants.
5. They also form an essential component of bile and help in the regulation of serum cholesterol.
6. They also have a role in blood coagulation, lipid absorption in intestine and transport of lipids from the intestine.
7. They also act as a cofactor in the activity of the enzyme lipases.

Classification of Phospholipids

Phospholipids are classified into several groups (Table 3.2).

Table 3.2 Classification of phospholipids

Glycerophospholipids
- Lecithins (phosphatidylcholine)
- Cephalins (phosphatidylethanolamine and phosphatidylserine)
- Phosphatidylinositols
- Cardiolipins and plasmalogens

Sphingolipids
- Sphingomyelins
- Cerebrosides
- Gangliosides

Glycerophospholipids

Glycerophospholipids, also called **phosphoglycerides**, are the derivatives of glycerol. They form major component of a biological membrane. Various glycerophospholipids include lecithin (phosphatidylcholine), cephalins (phosphatidylethanolamine and phosphatidylserine), phosphatidylinositol, etc. They are the derivatives of phosphatidic acid.

Phosphatidic acid: It consists of a molecule of glycerol, two fatty acids and a molecule of phosphoric acid.

- Phosphoric acid is attached at carbon-3 of glycerol. This is referred to as glycerol-3-phosphate.
- Alcoholic groups at carbons 1 and 2 of glycerol are esterified with two fatty acids. Usually, a saturated fatty acid is attached at carbon-1 (α-carbon) while a polyunsaturated fatty acid is attached at carbon-2 (β-carbon) of glycerol (Fig. 3.6).

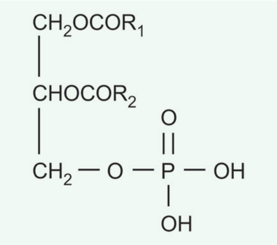


Fig. 3.6: Phosphatidic acid

Lecithins

Lecithins contain choline, a nitrogenous base, which is attached to phosphoric acid that is linked to the alcoholic group of glycerol at carbon-3.

- Lecithins are surface active agents and help in the emulsification of fat.
- They are widely distributed in the brain, nerve cells, sperm and egg yolk.
- Various hydrolytic enzymes, called **phospholipases**, act at different sites on lecithins and hydrolyze them. For example, phospholipase A_2 removes fatty acid from the second carbon atom. The leftover compound is called **lysolecithin** (Fig. 3.7).

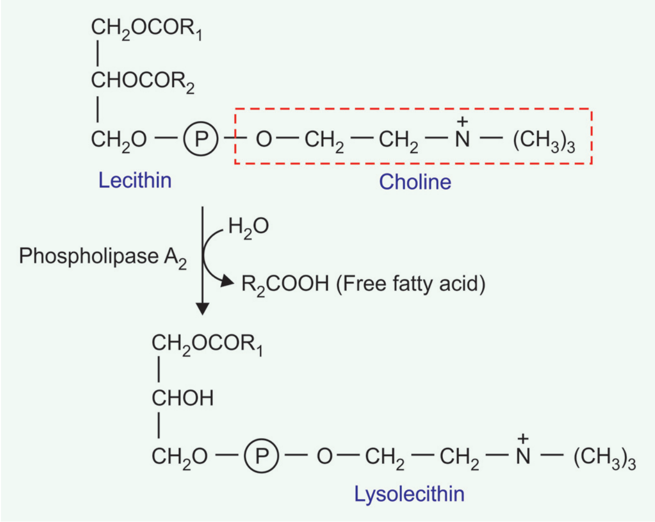

CH_2OCOR_1
$CHOCOR_2$
$CH_2O - (P) - O - CH_2 - CH_2 - \overset{+}{N} - (CH_3)_3$
Lecithin
Choline
Phospholipase A_2
H_2O
R_2COOH (Free fatty acid)
CH_2OCOR_1
CHOH
$CH_2O - (P) - O - CH_2 - CH_2 - \overset{+}{N} - (CH_3)_3$
Lysolecithin

Fig. 3.7: Hydrolysis of a lecithin to lysolecithin by phospholipase A_2

Cephalins

Cephalins are the glycerophospholipids which contain ethanolamine or serine, instead of choline (present in lecithins), as a nitrogenous base. Cephalins are present in erythrocytes membrane, brain and many other tissues.

Phosphatidylinositols

Phosphatidylinositols contain cyclic hexahydroxy-alcohol, called inositol, instead of a nitrogenous base. These are found, mainly, in the brain.

Plasmalogens

Plasmalogens contain an unsaturated fatty acid which is attached to the alcoholic group at carbon-1 of glycerol by an ether linkage, instead of a saturated fatty acid, which is linked by the ester linkage. Plasmalogens are present in the cardiac and skeletal muscles, and semen.

Sphingolipids

Sphingolipids do not contain glycerol, but are the derivatives of an unsaturated amino alcohol, called **sphingosine**.

Sphingosine has 18 carbon atoms and one double bond.

Ceramides: An N-acyl derivative of sphingosine is called ceramide. Ceramides are the parent compounds of most of the sphingolipids, i.e. sphingomyelins, cerebrosides and gangliosides.

Sphingomyelins

Sphingomyelins are the ceramides, which also contain phosphocholine or phosphoethanolamine. Myelin sheath is rich in sphingomyelins.

Cerebrosides

Cerebrosides are the ceramides, which are linked to a monosaccharide, such as galactose or glucose. Hence they may also be called **ceramide monosaccharides**. They are also referred to as **glycosphingolipids**. Most prevalent cerebrosides include **galactocerebrosides** and **glucocerebrosides**.

- Cerebrosides are named with respect to the presence of the fatty acid, e.g. **cerebron**. It is a galactosylceramide, which contains cerebronic acid.
- **Nervon** has an unsaturated derivative of lignoceric acid, referred to as nervonic acid.
- Cerebrosides are found in white matter of the brain and myelin sheath of nerves.

Gangliosides

Gangliosides are the most complex glycolipids. These are the **ceramides, which are linked to some oligosaccharide that has hexosamine**, such as glucosamine or galactosamine. In addition, gangliosides also have at least one molecule of **N-acetylneuraminic acid** (NANA, also called sialic acid).

- More than 60 gangliosides are known to occur.
- They are designated as GM_1, GM_2, GM_3, etc.
- Each one of it has a different oligosaccharide head group.
- They are present in large amount in ganglion cells of the central nervous system, particularly, in the nerve endings.

CHOLESTEROL

Cholesterol is a derivative of the **cyclopentanoperhydro-phenanthrene ring**, also called **steroid**

nucleus, which consists of four fused, nonpolar rings that are labeled as A, B, C and D.

- Cholesterol has **27 carbon atoms** with a **hydroxyl group on carbon number 3**, a **double bond between carbon numbers 5 and 6**, two **methyl groups** attached to **carbon numbers 18 and 19**, and a **side chain** that is attached to **carbon number 17** (Fig. 3.8).
- Because of the presence of the –OH group, cholesterol is a **steroid alcohol**, also called sterol.
- Due to the presence of this –OH group, cholesterol reacts with a long chain fatty acid and forms cholesterol ester.

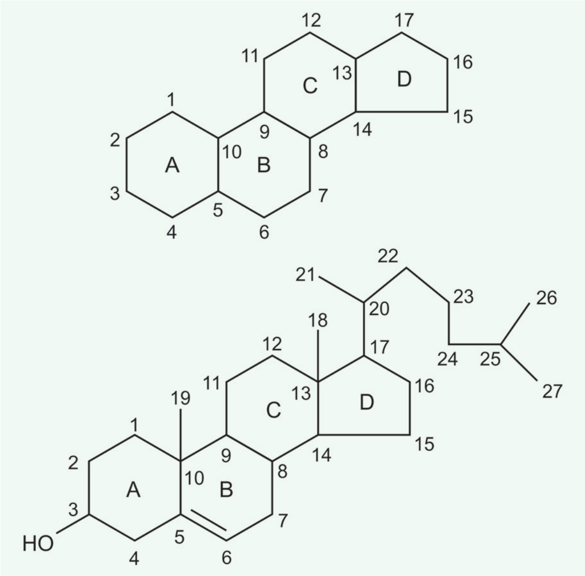


Fig. 3.8: Structure of the steroid ring and cholesterol

LIPOPROTEINS

As lipids are not soluble in the aqueous plasma, hence, they are transported in the body in association with proteins. These protein–lipid complexes are called **lipoproteins** or proteolipids. They **are globular micelle-like particles consisting of** nonpolar core of **triacylglycerols** and **cholesterol esters**, which are surrounded by the amphiphilic coating that consists of **proteins**, **cholesterol** and **phospholipids**.

CLASSIFICATION

Lipoproteins are classified into different groups, as follows:

Chylomicrons

These are very large particles that are secreted into lymphatics by mucosal cells of small intestine. About 90% of their mass is triglycerides, which together with cholesterol esters and fat-soluble vitamins form a core of the particle. Its surface layer is made of free cholesterol, phospholipids and apoproteins (apo B-48, apo A-I and apo A-IV).

Very Low Density Lipoproteins

Very low density lipoproteins (VLDL) are large particles, which are secreted into the blood stream by the hepatocytes. Nearly 50 to 60% of their mass is triacylglycerols. They contain relatively more cholesterol esters than chylomicrons. Their surface coat contains free cholesterol and phospholipids, a single molecule of apo B-100 and apo C.

VLDL is transformed into **intermediate density lipoproteins** (IDL). These are referred to as IDL (also called LDL_1) since their density as well as lipid composition are in between the VLDL and the LDL. IDL is either removed by liver or converted to low density lipoprotein (LDL).

Low Density Lipoproteins

Low density lipoprotein (LDL) has a density range of 1.019 to 1.063. It contains a single molecule of apo B-100, and carries about 75% of total cholesterol in human plasma. Its major function is to transport cholesterol from liver to the extrahepatic tissues.

High Density Lipoproteins

High density lipoprotein (HDL) fraction is very heterogeneous. Major apoproteins found in HDL include apo A-I, apo A-II, apo A-IV and apo C.

Various lipoproteins can be separated according to their density by ultracentrifugation. They can also be separated according to their protein content by electrophoresis (Table 3.3).

Apolipoproteins

Protein components of lipoproteins are called **apolipoproteins** or **apoproteins**. They serve as the structural components of lipoproteins, provide recognition sites for cell-surface receptors and serve as activators for enzymes involved in lipoprotein metabolism.

Various apolipoproteins are divided into different classes from A to E. At least nine of them are known to occur in human serum lipoproteins. They are referred to as A-I, A-II, B-48, B-100, C-I, C-II, C-III, D and E. All of them except B-100 are water soluble.

Apolipoprotein B-100 (apo B-100) is one of the largest monomeric proteins, which is synthesized by the liver and is present in the LDL.

Table 3.3 Properties and functions of lipoproteins

Lipoprotein class	Density on ultracentrifugation	Diameter (nm)	Protein/ lipid (%)	Major apoproteins	Major lipids	Source	Functions
Chylomicrons	<0.95	100–1000	2/98	B-48, A	TG	Small intestine	Transport dietary TG from intestine to extrahepatic tissues
Very low density lipoproteins (VLDL)	0.95–1.006	30–80	10/90	B-100, C	TG	Liver	Transport hepatic TG to extrahepatic tissues
Low density lipoproteins (LDL)	1.006–1.063	18–28	25/75	B-100	CHOL	Circulating VLDL and IDL	Transport CHOL to various tissues
High density lipoproteins (HDL)	1.063–1.21	10–20	40/60	A, C	CHOL, PL	Liver	Reverse CHOL transport from peripheral tissues to liver

TG: Triacylglycerol; CHOL: Cholesterol; PL: Phospholipids; FFA: Free fatty acids

PROPERTIES OF LIPIDS

Hydrolysis

Hydrolysis refers to the breakdown of a substance by the addition of water. Chemically, neutral fats are esters, so they are liable to hydrolysis. In the body, triacylglycerols are hydrolyzed by the enzymes lipases, which, in turn, release free fatty acids and glycerol (Fig. 3.9).

This can also occur by the non-enzymatic process. Whereas, partial hydrolysis of triglycerides yields monoglycerides, diglycerides and free fatty acids, complete hydrolysis yields glycerol and free fatty acids.

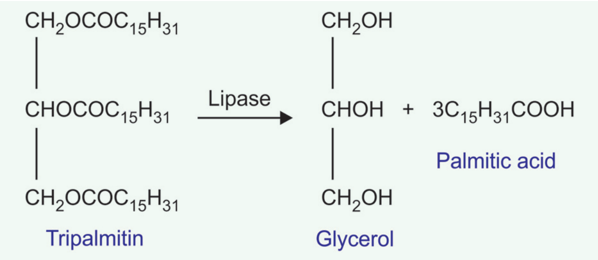


Fig. 3.9: Hydrolysis of a triacylglycerol by lipase

Saponification

Hydrolysis of triglyceride with aqueous sodium hydroxide yields glycerol and the sodium salts of fatty acids called **soaps**. This process is called **saponification**. Soaps are used as emulsifying agents.

Number of milligrams of KOH required to saponify free and combined fatty acid in one gram of a given fat is called its **saponification number**.

Triglyceride + Sodium hydroxide (or potassium hydroxide) ⟶ Glycerol + Soap

Rancidity

Rancidity is a general term that refers to the spoilage of food in such a way that it becomes undesirable (and usually unsafe) for consumption. It is a result of the hydrolysis and/or auto-oxidation of unsaturated fatty acids into short chain aldehydes and ketones, which give unpleasant odor and taste. Antioxidants such as vitamin E, prevent rancidity.

ANTIOXIDANTS

An antioxidant is a molecule that inhibits the oxidation of various substances.

- Oxidation is a chemical reaction that produces free radicals, leading to chain reactions that may damage cell. Antioxidants such as thiols and ascorbic acid (vitamin C), terminate chain reactions.
- Antioxidants include two different groups of substances:
  1. **Natural chemicals** which are found in food and body tissues. They have beneficial health effects. To balance the oxidative state, plants and animals

have a complex system of overlapping antioxidants, such as glutathione and enzymes (e.g. catalase and superoxide dismutase), which are present in the cell as well as dietary antioxidants such as vitamin A, vitamin C and vitamin E.

2. **Industrial chemicals** which are added to certain products, to prevent oxidation. These are called industrial antioxidants. They have diverse uses such as food and cosmetics preservatives, and act as inhibitors of rubber or gasoline deterioration.

LIPID PEROXIDATION

Lipid peroxidation is the **oxidative degradation of lipids**. It is the process in which **free radicals remove electrons** from lipids in cell membranes, resulting in **cell damage**.

- The process proceeds by **free radical chain reaction** mechanism.
- Lipid peroxidation affects **polyunsaturated fatty acids** (PUFA), because they contain multiple double bonds.
- The reaction consists of three steps, i.e. **initiation**, **propagation** and **termination**.
- Chemical products of oxidation are referred to as lipid peroxides or **lipid oxidation products** (LOPs).
- The end products of lipid peroxidation are **reactive aldehydes** such as malondialdehyde (**MDA**) and 4-hydroxynonenal (**HNE**).
  - If not terminated, it may **damage the cell membrane**. Phototherapy may cause hemolysis by rupturing red blood cell membranes.
  - In addition, end products of lipid peroxidation may be **mutagenic** and **carcinogenic**. For example, end product of MDA reacts with deoxyadenosine and deoxyguanosine in DNA, and form **DNA adducts**.

Important Questions

1. What are lipids? Classify them. Give biological functions of lipids.
2. What are fatty acids? Classify them. Write biological functions of polyunsaturated fatty acids.
3. Describe briefly:
   i. Classification of lipids with suitable examples
   ii. Plasmalogens
   iii. Sphingolipids
   iv. Chemistry and functions of cholesterol
   v. Cerebrosides
4. Write notes on:
   i. PUFA
   ii. Triacylglycerols
   iii. Lecithins
   iv. Lipoproteins
   v. Phospholipids
   vi. Apoproteins
   vii. Saponification
   viii. Rancidity
   ix. Antioxidants
   x. Lipid peroxidation

Chapter

4

Proteins and Amino Acids

PROTEINS

Protein (Greek: *proteios* means primary) is the class of natural products, which contains nitrogen and forms an essential structural component of a cell. They are most abundant and functionally diverse molecules present in all living systems.

- All **proteins**, from whatever source it may be, are the **polymers of amino acids**.
- **Amino acids are the hydrolyzed products of proteins**, they are also used in protein synthesis.
- Though a large number of amino acids may be found in the body, only **twenty of them are commonly occur in all mammalian proteins**.
- Each polypeptide has a **free α-amino group** on the **left-hand** side, which is referred to as the **N-terminal (amino terminal) end**. A peptide also has a free **α-carboxylic group** on the **right-hand** side, which is referred to as the **C-terminal (carboxyl terminal) end**.
- Large polypeptides are referred to as proteins (Fig. 4.1).

$$H_2NAA_1 - AA_2 - AA_3 \text{ - - - - - - - - - - } AA_{n-1} - AA_nCOOH$$

N-terminal end C-terminal end

Fig. 4.1: A polypeptide

Biological Functions of Proteins

Proteins serve a variety of functions (Table 4.1):

Table 4.1 Biological functions of proteins

Biological function	Examples of proteins
As structural proteins	Collagen, keratins, etc.
As enzymes	Pepsin, amylase, etc.
As hormones	Insulin
As transporters	Hemoglobin
As receptors	Hormone receptors
As storage molecules	Ferritin
In immune response	γ-Globulin

1. **Structural proteins:** Some of the proteins, e.g. **collagens** (found in bone and cartilage), **keratins** (found in hair and nail), etc., form an essential part of the particular structure within the body.
2. **Enzymes:** Some of the proteins, such as **pepsin**, catalyze biological reactions and act as enzymes.
3. **Hormones:** Some of the proteins act as hormones, e.g. **insulin**. They regulate metabolic processes within the cell.
4. **Transporters:** Several proteins serve as a carrier for the transport of a substance within the body. For example, **hemoglobin is important** for the transport of oxygen, **ceruloplasmin** transports copper, etc.
5. **Protein receptors:** Some of the proteins, such as **hormone receptors**, act as receptors for certain hormones and play an important role in signal transduction.
6. **Storage of substances:** Some of the proteins bind to a substance for its storage in different tissues in the body, e.g. **ferritin**. Ferritin stores Fe^{3+}.
7. **Antibodies:** Some proteins, such as **γ-globulin**, act as antibodies and provide immunity.

CLASSIFICATION OF PROTEINS

Depending upon their **solubility** and **physical properties**, proteins are classified into three groups (Table 4.2).

Table 4.2 Classification of proteins based on solubility and physical properties

Simple proteins	Conjugated proteins	Derived proteins
• Albumins • Globulins • Prolamines • Glutelins • Scleroproteins • Histones	• Nucleoproteins • Glycoproteins • Phosphoproteins • Metalloproteins • Lipoproteins	• Primary derived proteins • Secondary derived proteins

1. Simple Proteins

Based on their solubility, proteins are divided into different groups, as follows:

- **Albumins:** Albumins are soluble in water and neutral salt solutions. For example, serum albumin, lactalbumin, egg albumin, etc.
- **Globulins:** These are sparingly soluble in water, but are soluble in a neutral salt solution. Generally, globulins are found with albumins, e.g. serum globulins, lactglobulin, egg globulin, etc.
- **Prolamines:** Prolamines are insoluble in water, but are soluble in 60–80% of alcohol. For example, gliadin of wheat, zein of maize, etc.
- **Glutelins:** Glutelins are soluble in dilute acid or alkali, e.g. gluten of wheat, oryzenin of rice, etc.
- **Scleroproteins:** These are highly insoluble and are found, mostly, in the supporting tissues such as bone and cartilages, and in protective tissues like skin, hair, nail, etc.
- **Histones:** Histones are soluble in water. They are rich in basic amino acids, e.g. nucleohistones.

2. Conjugated Proteins

These are the proteins, which are conjugated with some nonprotein substance called prosthetic group. These include:

- **Nucleoproteins:** Proteins are conjugated with nucleic acids, e.g. histones.
- **Glycoproteins:** Proteins are conjugated with carbohydrates, e.g. mucin.
- **Phosphoproteins:** Proteins are conjugated with phosphoric acid, which is attached through the –OH group of serine, threonine or tyrosine, e.g. casein (a milk protein).
- **Metalloproteins:** Proteins are associated with some metal. They are also called metal-containing proteins. For example, ceruloplasmin (copper-containing protein), hemoglobin (iron-containing protein), etc.
- **Lipoproteins:** Proteins conjugated with lipids, e.g. chylomicrons, very low density lipoproteins (VLDL), etc.

3. Derived Proteins

These are the derivatives of simple proteins. They include:

- **Primary derived proteins:** These are obtained as a result of denaturation or coagulation of a protein.
- **Secondary derived proteins:** These are obtained as a result of the hydrolysis of proteins, i.e. by the cleavage of the peptide bonds.

STRUCTURAL ORGANIZATION OF PROTEINS

Variations in the number and order of amino acids, i.e. the manner in which various amino acids are arranged in a polypeptide chain, reflect structural organization of a protein. There are four levels of protein structure:

1. Primary Structure of Proteins

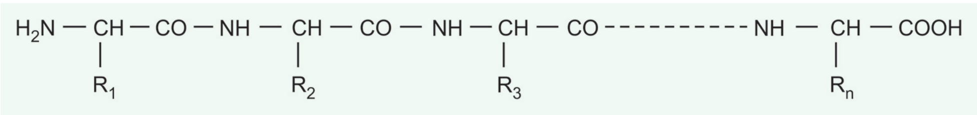
Primary structure of a protein refers to the **order and sequence of amino acids** in a protein chain.

- Protein with a primary structure is a single polypeptide chain that has N-terminal and C-terminal ends, and various amino acids are linked together by the peptide linkages (Fig. 4.2).
- Some of the polypeptides may be synthesized as large peptides which are cleaved into two or more small peptides. These peptides may be linked together by the disulfide bonds, e.g. insulin.
- Thus, primary structure of a protein has peptide linkages and disulfide bonds. A change in the sequence or number of amino acids may cause some abnormality, which in turn may also affect functions and properties of the protein.

2. Secondary Structure of Proteins

Secondary structure of a protein refers to the folding and twisting patterns of the polypeptide chain, i.e. spatial arrangement of the polypeptide. Secondary structures include α-helix and β-sheet structures.

Helix structure: As we know, helix is formed by twisting the backbone of the straight chain of a polypeptide.



$$H_2N-\underset{\underset{R_1}{|}}{CH}-CO-NH-\underset{\underset{R_2}{|}}{CH}-CO-NH-\underset{\underset{R_3}{|}}{CH}-CO\text{----------}NH-\underset{\underset{R_n}{|}}{CH}-COOH$$

Fig. 4.2: Primary structure of a protein

α-Helix

It refers to the right-handed folding of the protein chain (Fig. 4.3).

- It is the most stable conformation, which is observed in a peptide chain, e.g. keratin.

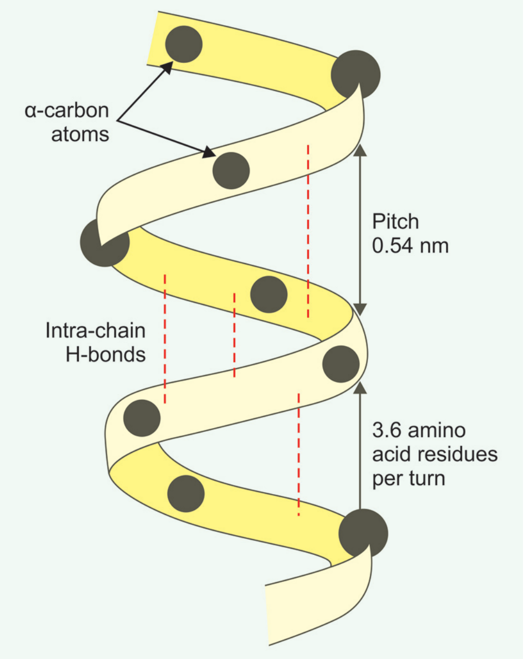


Fig. 4.3: Secondary structure of protein: α-helix (right-handed helix)

- There are 3.6 amino acid residues per turn of the helix.
- As a result of formation of the α-helix, several amino acids, which are present in the side chain of the acidic or basic amino acids, come closer to each other and form hydrogen bonds between the –CO and the –NH groups of these amino acids. These are referred to as **intrachain peptide linkages**.

β-Pleated Sheet Structure

The second regular structural organization found in proteins is referred to as β-pleated sheet structure. It is referred to as β, since it was described after the discovery of the α-helix structure.

- **β-Pleated sheet structure** has rippled or pleated edge, therefore, this conformation is also called pleated sheet-like structure.
- Proteins having β-sheet structure, e.g. wool, may contain 2 to 12 polypeptide strands.
- β-Pleated sheet-like structure is formed due to the stretching of the polypeptide chains. As a result of formation of the pleated edges, several acidic or basic amino acids of the neighboring polypeptide chains may come closer to each other and form hydrogen bonds between the –CO and the –NH groups of these amino acids. These are referred to as **interchain peptide linkages** (Fig. 4.4).

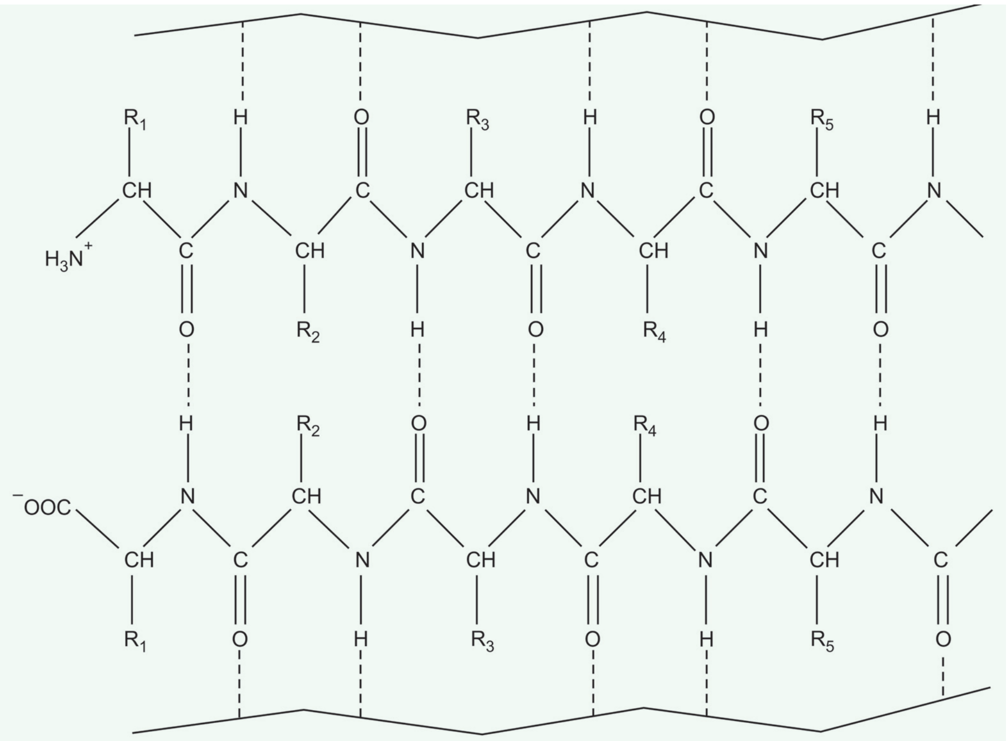


Fig. 4.4: Secondary structure of protein: β-pleated sheet

- In a pleated sheet structure, polypeptide chains may run in parallel or antiparallel directions:
  - **Parallel β-sheet** is formed when the two polypeptide chains run in the same direction.
  - **Antiparallel β-sheet** is formed when the two polypeptide chains run in opposite directions.
- Various forces which stabilize secondary structure of a protein include **hydrogen bonds**, **hydrophobic interactions**, **electrostatic interactions** and **van der Waals forces**.

3. Tertiary Structure of Proteins

Tertiary structure of a protein refers to the intramolecular folding of the polypeptide chain, thereby forming a compact three-dimensional structure that has a specific shape.

- **Electrovalent linkages**, **hydrogen bonds**, **disulfide bridges**, **van der Waals forces** and **hydrophobic interactions** are responsible for the stabilization of the tertiary structure of proteins (Fig. 4.5).

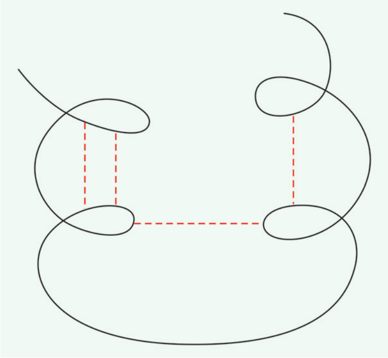


Fig. 4.5: Tertiary level of protein structure

Depending upon their overall morphology, proteins having tertiary level of structures may exhibit globular or fibrous shapes.

Globular Proteins

Globular proteins have a spheroidal shape with varying sizes. They are highly water soluble and perform various functions such as catalysis of a substance, transport of substances, regulation of metabolic pathways and gene expression. Their shape is the result of the complex interaction between the secondary, tertiary and sometimes may be quaternary structures.

Both types of the secondary structures, i.e. the α-helices and β-sheets, may occur in globular proteins, in varying proportions and combinations. Some of the globular proteins, such as cytochromes, consist of only α-helices while others such as immunoglobulins, have a large proportion of β-sheets. Most of the other globular proteins such as lactate dehydrogenase, have significant amount of both types of the secondary structures.

Most common globular proteins include hemoproteins such as hemoglobin, myoglobin, cytochromes, catalase, etc.

Fibrous Proteins

Fibrous proteins exhibit special mechanical properties. They contain large amount of the regular secondary structures and have long cylindrical (rod-like) shapes.

- They have low solubility in water.
- Fibrous proteins have protective, connective or supportive role in the living organisms, e.g. collagen, keratin, tropomyosin, etc.
- They are found as the components of skin, connective tissue, blood vessel walls, and sclera and cornea of the eye.

4. Quaternary Structure of Proteins

Proteins having quaternary structure have several identical and/or different peptide chains. Each of its peptide chain is referred as a **subunit**. Several subunits are associated with a specific geometry (Fig. 4.6).

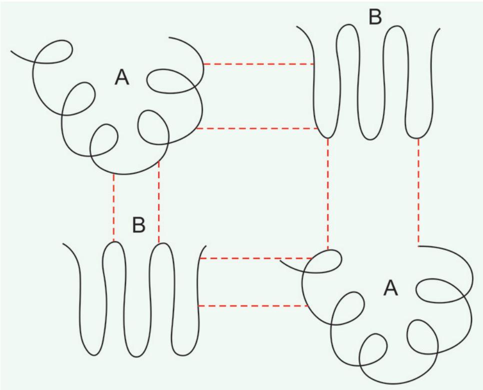


Fig. 4.6: Quaternary level of protein structure

Oligomeric Proteins

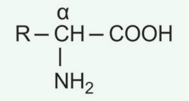
Proteins with more than one subunit are called oligomeric proteins, e.g. hemoglobin. Hemoglobin is an oligomeric protein. It has four subunits, i.e. 2α and 2β chains ($\alpha_2\beta_2$). Disintegration of the monomeric subunits of the protein having quaternary structure may result in loss of its biological activity.

AMINO ACIDS

As mentioned above, amino acids are the hydrolyzed products of proteins. These are also used in the synthesis of proteins. All the naturally occurring amino acids are designated as **α-amino acids**, because:

- Each amino acid has a **carboxyl group (–COOH)** as well as a primary **amino group (–NH$_2$)**, which are present on the α-carbon atom.
- Each amino acid also has an atom or group, which is attached to the α-carbon atom. It is called **R-group**.

  General structure of an amino acid is shown in Fig. 4.7.



$$\mathrm{R-\overset{\alpha}{C}H(NH_2)-COOH}$$

Fig. 4.7: General structure of an amino acid

BIOLOGICAL FUNCTIONS OF AMINO ACIDS

1. Amino acids are important **dietary source of nitrogen**.
2. They are also used in **protein biosynthesis**.
3. Some of the amino acids, referred to as glucogenic amino acids, also serve as a **source of energy**.
4. Sulfur-containing amino acids serve as a **source of sulfur** for the body.
5. Besides, several amino acids are also used in the biosynthesis of some **specialized products**, e.g.
   i. **Glycine** is used in the synthesis of **creatine**, **glutathione**, **heme**, **bile acids** and the **purine ring**.
   ii. **Cysteine** is used in the biosynthesis of **glutathione**.
   iii. **Methionine** is important in **one-carbon metabolism**.
   iv. **Aromatic amino acids** are used in the synthesis of **thyroid hormones, catecholamines** (dopamine, norepinephrine and epinephrine; hormones of the adrenal medulla) and **melanin** (a brown-black pigment present in skin and iris).
   v. **Aspartate** and **glutamine** are used in the synthesis of **purines** and **pyrimidines**.
6. Some of the amino acids, like glycine and cysteine, are used in the detoxification reactions.

CLASSIFICATION OF AMINO ACIDS

According to the properties of the side chain such as charge or polarity, amino acids may be classified into several groups

ACCORDING TO CHARGE

According to the net ionic charge of its acidic and basic groups, amino acids may be classified as neutral, acidic or basic (Table 4.3).

Table 4.3 Classification of amino acids according to charge

Amino acid	Abbreviation*
NEUTRAL AMINO ACIDS	
Simple amino acids	
Glycine	Gly
Alanine	Ala
Branched chain amino acids	
Valine	Val
Leucine	Leu
Isoleucine	Ile
Hydroxy amino acids	
Serine	Ser
Threonine	Thr
Aromatic amino acids	
Phenylalanine	Phe
Tyrosine	Tyr
Tryptophan	Trp
Sulfur-containing amino acids	
Cysteine	Cys
Methionine	Met
ACIDIC AMINO ACIDS	
Aspartic acid	Asp
Glutamic acid	Glu
Amides of the acidic amino acids	
Asparagine	Asn
Glutamine	Gln
BASIC AMINO ACIDS	
Lysine	Lys
Arginine	Arg
Histidine	His
IMINO ACID	
Proline	Pro

* Three letters abbreviation is widely used. This, generally, includes first three letters of an amino acid.

1. Neutral Amino Acids

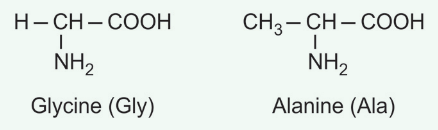
These amino acids have one amino group (–NH$_2$) and one carboxyl group (–COOH). Thus, they may also be referred to as **monoamino-monocarboxylic acids**. They are neutral in reaction. These amino acids may be aliphatic or aromatic in nature.

Aliphatic Amino Acids

Aliphatic amino acids are further subdivided into various groups, as follows:

Simple aliphatic amino acids

These include **glycine**, which has hydrogen (H), and **alanine**, which has a methyl group ($-CH_3$) as the R-group (Fig. 4.8).

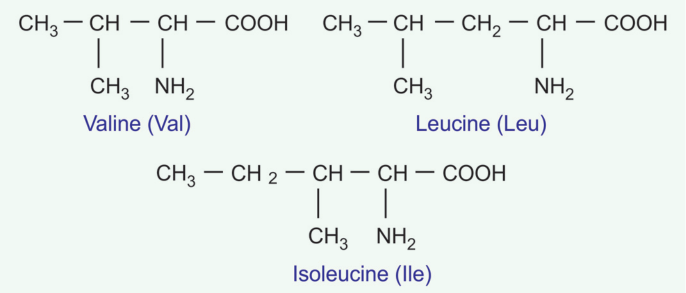


$\mathrm{H-\underset{\underset{NH_2}{|}}{CH}-COOH}$ $\qquad$ $\mathrm{CH_3-\underset{\underset{NH_2}{|}}{CH}-COOH}$

Fig. 4.8: Glycine and alanine

Branched chain amino acids

These amino acids have an aliphatic side chain, which is not straight, but is branched. These include **valine**, **leucine** and **isoleucine** (Fig. 4.9).



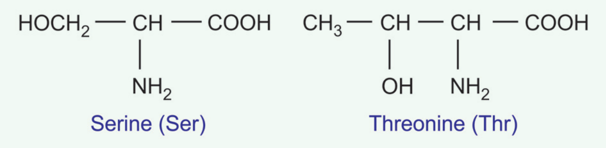
$\mathrm{CH_3-\underset{\underset{CH_3}{|}}{CH}-\underset{\underset{NH_2}{|}}{CH}-COOH}$ $\qquad$ $\mathrm{CH_3-\underset{\underset{CH_3}{|}}{CH}-CH_2-\underset{\underset{NH_2}{|}}{CH}-COOH}$

$\mathrm{CH_3-CH_2-\underset{\underset{CH_3}{|}}{CH}-\underset{\underset{NH_2}{|}}{CH}-COOH}$

Fig. 4.9: Branched chain amino acids

Hydroxyl group-containing amino acids

These amino acids have a hydroxyl (–OH) group in the aliphatic side chain (R group). These include **serine** and **threonine** (Fig. 4.10).



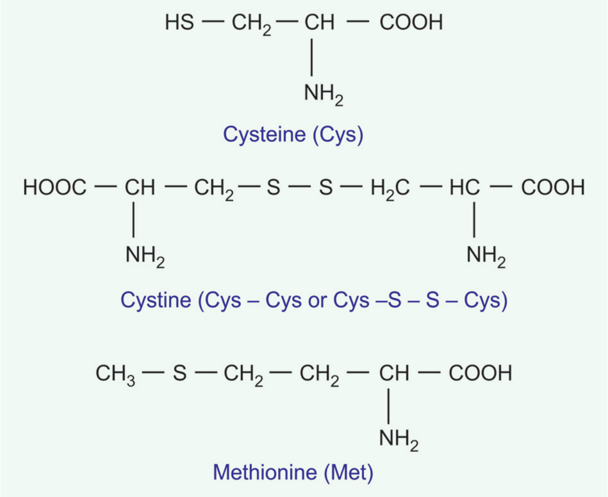
$\mathrm{HOCH_2-\underset{\underset{NH_2}{|}}{CH}-COOH}$ $\qquad$ $\mathrm{CH_3-\underset{\underset{OH}{|}}{CH}-\underset{\underset{NH_2}{|}}{CH}-COOH}$

Fig. 4.10: Hydroxyl group-containing amino acids

Sulfur-containing amino acids

In addition to C, H, O and N, these amino acids also contain sulfur (S) in their side chain (R group). These include **cysteine** and **methionine**.

- **Cysteine** has a thiol group (–SH) at the terminal point of the R group.
- Two molecules of cysteine, when present at the nearby positions within the same peptide (intra-chain) or two different peptide chains (interchain), can link with each other through their –SH groups and form another amino acid called **dicysteine**, which has a disulfide linkage (–S–S–). Dicysteine is also referred to as **cystine**.
- **Methionine** has a methyl group attached to sulfur (CH_3–S–) at the terminal point in R group (Fig. 4.11).



$\mathrm{HS-CH_2-\underset{\underset{NH_2}{|}}{CH}-COOH}$

$\mathrm{HOOC-\underset{\underset{NH_2}{|}}{CH}-CH_2-S-S-H_2C-\underset{\underset{NH_2}{|}}{HC}-COOH}$

$\mathrm{CH_3-S-CH_2-CH_2-\underset{\underset{NH_2}{|}}{CH}-COOH}$

Fig. 4.11: Sulfur-containing amino acids

Aromatic Amino Acids

These amino acids have an aromatic ring in the side chain. Aromatic amino acids include **phenylalanine** (alanine attached to a phenyl group), **tyrosine** (4-hydroxyphenylalanine, also called p-hydroxy-phenylalanine) and **tryptophan** (alanine attached to an indole ring; Fig. 4.12).

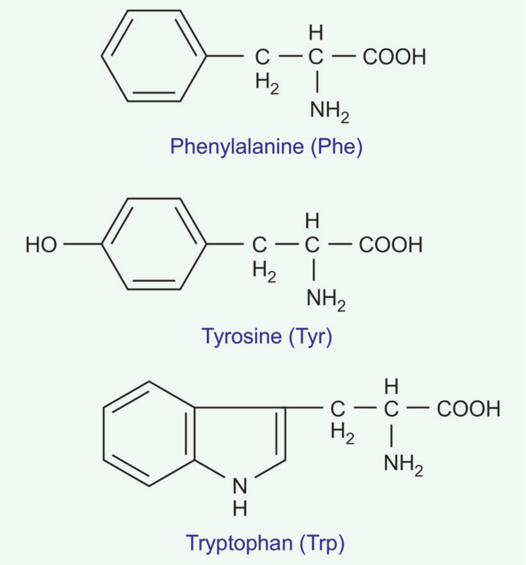


Fig. 4.12: Aromatic amino acids

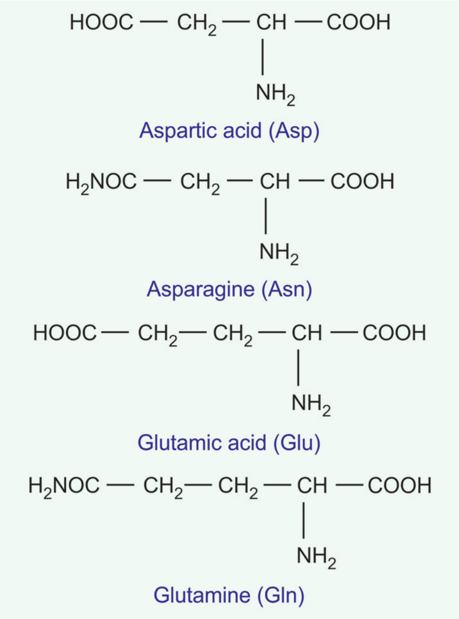
2. Acidic Amino Acids

Monoamino-dicarboxylic Acids

These amino acids have **one amino group** but **two carboxyl groups**. Hence, these amino acids can also be referred to as monoamino-dicarboxylic acids. These include **aspartic acid** and **glutamic acid**.

Amides of the Acidic Amino Acids

There are two amino acids which are actually not acidic in reaction but are the amides of the acidic amino acids. These include **asparagine** and **glutamine**. In these amino acids, an additional amino group is attached to the terminal carboxyl group of the acidic amino acid and forms an amide group (Fig. 4.13).



$HOOC-CH_2-CH(NH_2)-COOH$

$H_2NOC-CH_2-CH(NH_2)-COOH$

$HOOC-CH_2-CH_2-CH(NH_2)-COOH$

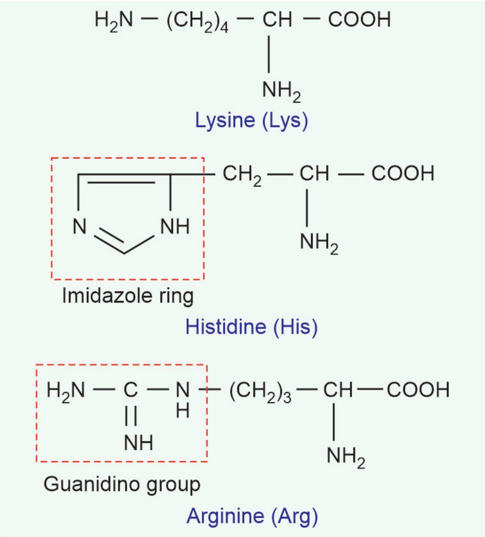
$H_2NOC-CH_2-CH_2-CH(NH_2)-COOH$

Fig. 4.13: Acidic amino acids (aspartic acid and glutamic acid) and their amides (asparagine and glutamine)

3. Basic Amino Acids

They have one carboxyl group and either **more than one amino group** in the aliphatic side chain or a **heterocyclic ring** that is basic in reaction. These include **lysine**, **arginine** and **histidine**.

- **Lysine** has two $-NH_2$ groups. One amino group is attached to the alpha carbon atom while the other one is attached as the terminal carbon atom of the aliphatic side chain.
- **Arginine** has a guanidino group which is basic in reaction.
- **Histidine** has a heterocyclic ring, called imidazole ring, which is basic in nature (Fig. 4.14).



$H_2N-(CH_2)_4-CH(NH_2)-COOH$

$H_2N-C(=NH)-NH-(CH_2)_3-CH(NH_2)-COOH$

Fig. 4.14: Basic amino acids

4. Imino Acid

In addition to the various amino acids, as discussed above, some of the proteins, such as collagen, contain an imino acid, referred to as **proline** (Fig. 4.15).

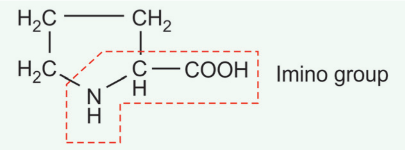


Fig. 4.15: Proline—an imino acid

- **Proline** does not contain a primary α-amino group ($-NH_2$) but has a secondary amino group (–NH–), called imino group, which forms a part of the heterocyclic ring. Hence, proline is not actually an amino acid but is an imino acid. However, for uniformity, proline is included in the list of the α-amino acids.
- **4-Hydroxyproline:** Proline is incorporated into the peptide chain during collagen synthesis. When the immature collagen, called protocollagen, undergoes post-translational modifications, some of its proline residues get hydroxylated and converted to **4-hydroxyproline** (hydroxyproline), which is found in collagen. Thus, in addition to proline, 4-hydroxyproline is also found in the blood.

ACCORDING TO POLARITY

According to polarity, amino acids are classified into different groups, as follows (Table 4.4):

Table 4.4 Classification of amino acids according to polarity

Nonpolar side chain	Polar side chain	
	Uncharged	Charged
- Glycine - Alanine - Valine - Leucine - Isoleucine - Methionine - Phenylalanine - Tryptophan - Proline	- Serine - Threonine - Asparagine - Glutamine - Tyrosine - Cysteine	- Lysine - Arginine - Histidine - Aspartate - Glutamate

1. Polar Amino Acids

These amino acids have a polar group in the side chain, which may be charged or uncharged.

- **Uncharged polar amino acids** have a polar side chain which does not bear any charge and is neutral in reaction. For example, –OH group-containing amino acids **serine**, **threonine** and **tyrosine**. Their –OH group also serves as a site for the attachment of phosphate, monosaccharide or oligosaccharide.
- **Charged polar amino acids** have a side chain which has a charge, e.g. **acidic amino acids** (aspartate and glutamate), their amides (asparagine and glutamine) and **basic amino acids** (lysine, arginine and histidine).

2. Nonpolar Amino Acids

They have a side chain, which is nonpolar. These amino acids neither release proton nor participate in hydrogen/ionic bond formation and are referred to as nonpolar amino acids. These are glycine, alanine, valine, leucine, isoleucine, methionine, phenylalanine, tryptophan and proline.

ACCORDING TO DIETARY REQUIREMENT

According to dietary requirement, amino acids are classified into three groups, referred to as essential, semi-essential and nonessential amino acids (Table 4.5).

1. Essential Amino Acids

All the amino acids cannot be synthesized in the body. Hence, some of them are to be included in the diet and are called essential or indispensable amino acids. There are eight amino acids, which are dietary essential for adult human beings. These include **methionine**, **threonine**, **tryptophan**, **valine**, **isoleucine**, **leucine**, **phenylalanine** and **lysine**.

Table 4.5 Classification of amino acids according to dietary requirement

Essential	Semi-essential	Nonessential
- Methionine - Threonine - Tryptophan - Valine - Isoleucine - Leucine - Phenylalanine - Lysine	- Arginine - Histidine	- Glycine - Alanine - Serine - Cysteine - Tyrosine - Aspartate - Glutamate - Asparagine - Glutamine - Proline

2. Semi-essential Amino Acids

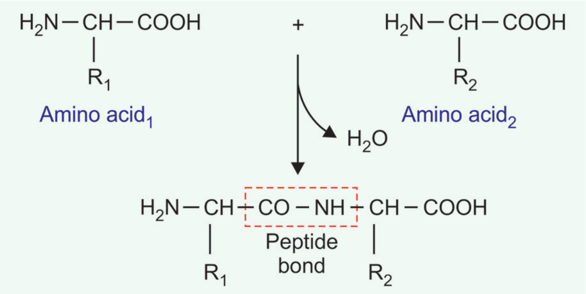
Besides the above eight amino acids, there are two amino acids, i.e. **arginine** and **histidine**, which are not essential for normal adult human beings but become dietary essential under certain conditions when either their demand is increased (such as during growth, pregnancy or lactation) or in hypercatabolic states (like high grade fever and chronic infections). Hence, these two amino acids are referred to as semi-essential amino acids.

3. Nonessential Amino Acids

All the remaining amino acids which can be synthesized by human beings are not dietary essential and are referred to as nonessential amino acids.

Formation of the Peptide Bond

Peptides are biologically occurring short chains of amino acid monomers, which are linked by peptide (amide) bonds. When two amino acids join together, they form a **peptide bond** (**–CO–NH–**). During this process, carboxyl group of one amino acid reacts with the amino group of the other amino acid. This is a condensation reaction and results in the formation of **amide linkage**, called peptide bond (Fig. 4.16).



$$\underset{\text{Amino acid}_1}{H_2N-\underset{R_1}{\underset{|}{CH}}-COOH} + \underset{\text{Amino acid}_2}{H_2N-\underset{R_2}{\underset{|}{CH}}-COOH} \xrightarrow{H_2O} H_2N-\underset{R_1}{\underset{|}{CH}}-\underset{\text{Peptide bond}}{\boxed{CO-NH}}-\underset{R_2}{\underset{|}{CH}}-COOH$$

Fig. 4.16: Formation of a peptide bond

The resulting molecule is called a **dipeptide**. Accordingly, several amino acids can join to each other and form a **polypeptide**. One end of the peptide has a free **carboxyl group**, which is referred to as **C-terminus** while the other end has a free amino group and is referred to as **N-terminus**.

BIOLOGICALLY IMPORTANT PEPTIDES

The shortest peptides are dipeptides, consisting of 2 amino acids, which are joined together by a single peptide bond, followed by tripeptides, tetrapeptides, etc. A polypeptide is a long, continuous and unbranched peptide chain.

Many peptides containing only a few amino acid residues **have important biological functions**, e.g. hormones or other regulatory molecules such as glutathione, oxytocin, etc.

Glutathione

Glutathione is a tripeptide (Glu-Cys-Gly) consisting of glutamate, cysteine and glycine. Glutathione reduces disulfide bond, by serving as an electron donor. In this process, glutathione is converted to its oxidized form, i.e. oxidized glutathione or glutathione disulfide (GSSG). Once oxidized, glutathione can be reduced back by glutathione reductase, using NADPH as an electron donor. The ratio of reduced glutathione to oxidized glutathione within cells is often used as a measure of cellular oxidative stress. Glutathione is capable of preventing damage to important cellular components caused by reactive oxygen species such as free radicals, peroxides, lipid peroxides and heavy metals.

Oxytocin and Vasopressin

These are the two peptide hormones consisting of 9 amino acids.

Oxytocin

It is a hormone, normally, produced by the paraventricular nucleus of the hypothalamus and released by the posterior pituitary. Oxytocin is released into the bloodstream in response to stretching of the cervix and uterus during labor, and with the stimulation of nipples during breastfeeding. This helps mother in bonding with the baby, and in milk production. As a therapeutic agent, it is used to cause contraction of the uterus, in order to start or increase the speed of labor and to stop bleeding following delivery.

Vasopressin

Vasopressin, also known as **antidiuretic hormone** (**ADH**), is a neurohypophysial hormone found in most mammals. In most species, it contains arginine and is thus also called **arginine-vasopressin** (**AVP**). Its two primary functions are to retain water in the body and to constrict blood vessels. Vasopressin regulates water reabsorption in the kidney's collecting ducts, the tubules which receive very dilute urine produced by the functional unit of the kidney, the nephrons. It also increases peripheral vascular resistance, which in turn increases arterial blood pressure. It plays a key role in homeostasis in the regulation of water, glucose and salts in the blood.

Bradykinin

Bradykinin is a physiologically and pharmacologically active peptide of the kinin group of proteins, consisting of nine amino acids. It is an inflammatory mediator, a peptide that causes blood vessels to dilate (enlarge) and, therefore, causes blood pressure to fall.

Angiotensinogen

It is a serum globulin produced in the liver, also known as renin substrate. Renin cleaves two amino acids from angiotensinogen and forms a decapeptide, called angiotensin I.

- **Angiotensin I** have no biological activity and exists solely as a precursor of angiotensin II.
- **Angiotensin II** is formed from angiotensin I with the removal of two C-terminal residues by the angiotensin-converting enzyme (ACE) within the lungs. It acts as a hormone and increases blood pressure. In addition, angiotensin II also acts at Na/H^+ exchanger in the proximal tubules of the kidney, to stimulate Na reabsorption and H^+ excretion, which is coupled to bicarbonate reabsorption. This ultimately results in an increase in blood volume, pressure and pH.

  Angiotensin II is degraded to angiotensin III by angiotensinases, located in red blood cells and the vascular beds of most tissues.
- **Angiotensin III** increases mean arterial pressure. It is further cleaved to angiotensin IV.
- **Angiotensin IV** is a hexapeptide that, like angiotensin III, has less activity.

PROPERTIES OF AMINO ACIDS AND PROTEINS

Isomerism

All the naturally occurring amino acids, except glycine, have at least one asymmetric carbon atom, hence are

optically active. All the naturally occurring amino acids occur as L-isomers.

Acid–Base Properties

Amino acids may acquire either positive or negative charge, hence exhibit acid–base properties.

- **At low pH**, an amino acid exists in the cationic form as its amino group is protonated, i.e. in the form of $–NH_3^+$.
- **As the pH rises**, carboxyl group loses its proton and the amino acid is present as an **ampholyte**.
- With **further increase in pH**, an amino group is deprotonated, thereby exhibiting the anionic form.

Isoelectric pH

As discussed above, the net charge on the molecule is affected by pH of its surrounding environment and can become more positively or negatively charged due to gain or loss of protons (H^+), respectively.

The isoelectric pH, is the pH at which a particular molecule carries no net charge. The standard nomenclature to represent the isoelectric pH is **isoelectric point** (**pI**). The pI value can affect solubility of a molecule at a given pH.

Such molecules have minimum solubility in water or salt solutions at the pH that corresponds to their pI and often precipitate out of solution.

Biological amphoteric molecules, such as proteins, contain both acidic as well as basic functional groups. Amino acids that make up proteins may be positive, negative, neutral or polar in nature and together give a protein its overall charge. At a pH below their pI, proteins carry a net positive charge while above their pI, they carry a net negative charge (Fig. 4.17).

Zwitterion

Zwitterion, formerly called a **dipolar ion**, is a neutral molecule with both positive and negative charges (in some cases multiple positive and multiple negative charges may be present). Unlike simple amphoteric compounds that may only form either a cationic or anionic species, a zwitterion, simultaneously, has both the ionic states. Amino acids are the best known examples of zwitterions. These compounds contain an ammonium and a carboxylate group, and can be viewed as arising via a kind of intramolecular acid–base reaction (Fig. 4.17).

Solubility

As a protein contains multiple charged groups, its solubility depends upon the concentration of the dissolved salt, polarity of the solvent, pH and temperature. Solubility of a protein, at low ion concentration, increases as salt is added, a phenomenon called **salting in**. However, after more salt is added, particularly, as sulfate salts (such as ammonium sulfate), the solubility of protein decreases. This is referred to as **salting out**. Since different proteins precipitate at different salt concentrations, salting out is the basis of the commonly used protein purification procedures.

Solubility is a property that can be used to classify proteins that result from the various levels of structure. For example, fibrous proteins such as keratin (the protein present in hair), collagen (a structural protein found in tendons and cartilage), myosin (a protein found in most muscle tissue) and fibrin (the protein that allows blood to clot) are not soluble in water. On the other hand, globular proteins, e.g. albumins are soluble in water.

The solubility of proteins in aqueous buffer depends on the distribution of hydrophilic and hydrophobic amino acid residues on the protein's surface. Hydrophobic residues predominantly occur in the globular protein core. Proteins that have high hydrophobic amino acid content on the surface have low solubility in an aqueous solvent. Charged and polar surface residues interact with ionic groups in the solvent and increase the solubility of a protein.

Molecular Weight of Proteins

Molecular weight is a dimensionless quantity, defined as the ratio of the particle mass to 1/12th the mass of a carbon atom. Molecular masses of proteins are expressed in units of **daltons** (**D**). One thousand D is equal to 1 **kilodalton** (kD or kDa). The size of a synthesized protein can be measured by the number of amino acids it contains and by its total molecular mass, e.g. yeast proteins are, on average, 466 amino acids long and **53 kDa** in mass.

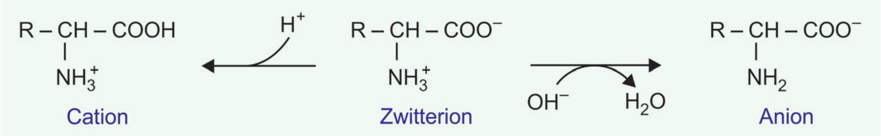


Fig. 4.17: Various ionic forms of an amino acid

Shape of Proteins

The function of a protein is determined by its shape, which, in turn, is determined by its **primary structure** (sequence of amino acids) and **folding of the chain**.

Proteins come in a wide variety of shapes, and are, generally, 50 to 2000 amino acids long. Large proteins, generally, consist of several distinct **protein domains**, structural units that fold more or less independently of each other. Folding of a protein chain is constrained by many different sets of **weak noncovalent bonds** that form between one part of the chain and another. These include **hydrogen bonds**, **ionic bonds** and **van der Waals attractions**. In addition, the **hydrophobic** interactions also play an important role.

Globular proteins are spherical, elliptical or oval in shape and soluble in water, acids and bases, while **fibrous proteins** are not soluble except in strong concentration of acid and alkali. Fibrous proteins are more rod-like in structure and are one of three types of scleroproteins that make up connective tissue, bone matrices, tendons and muscle fibre.

Acidic and Basic Proteins

Biological amphoteric molecules, such as **proteins**, contain both **acidic** and **basic functional groups**. Amino acids that make up proteins may be positive, negative, neutral or polar in nature and together give a protein its overall charge.

There are three amino acids that have **basic side chains** at neutral pH. These are arginine (Arg), lysine (Lys) and histidine (His). Their side chains contain nitrogen and resemble ammonia, which is a base. Their pKa's are high enough that they tend to bind protons, gaining a positive charge in the process.

Two amino acids have **acidic side chain** at neutral pH. These are aspartic acid or aspartate (Asp) and glutamic acid or glutamate (Glu). Their side chains have carboxylic acid groups whose pKa's are low enough to loose protons, becoming negatively charged in the process.

Histones are highly alkaline proteins found in eukaryotic cell nuclei that package and order the DNA into structural units, called nucleosomes. They are the chief protein components of chromatin and play a role in gene regulation.

Albumins are a family of globular proteins, the most common of which are serum albumins. All proteins of the albumin family are water soluble, moderately soluble in concentrated salt solutions and experience heat denaturation.

PRECIPITATION OF PROTEINS

Protein precipitation is widely used to concentrate proteins and purify them from various contaminants. The underlying mechanism of precipitation is to alter the solvent potential, more specifically, by lowering the solubility of the solute by addition of a reagent.

Protein precipitate formation occurs in a stepwise process.

First, **a precipitating agent is added** and the solution is steadily mixed. Mixing causes the precipitant and protein to collide. Next, **proteins undergo a nucleation phase**, where sub-microscopic sized proteins aggregate. Once the particles reach a critical size, they continue to grow by colliding into each other and sticking or flocculating. During the final step, the **precipitated particles repeatedly collide and stick**, then break apart, until a stable mean particle size is reached.

As mentioned above, proteins are charged, high molecular weight, colloidal particles and can be precipitated from a solution under several situations.

At the pI

At the pI the negative and positive charges cancel each other, repulsive electrostatic forces are reduced and the attraction forces predominate. The attraction forces will cause aggregation and precipitation. The pI of most of the proteins is in the range of pH 4–6. For example, precipitation of casein during cheese-making is an isoelectric precipitation.

Salting out

Proteins can be **precipitated by the addition of a salt solution**. This process is known as **salting out** (also known as antisolvent crystallization or precipitation crystallization). It is an effect based on the electrolyte–nonelectrolyte interaction, in which the nonelectrolyte could be less soluble at high salt concentration. It is commonly used as the method of separating proteins.

Salt concentration needed for the precipitation from the solution, differs from protein to protein.

- Highly soluble salts like NaCl or $(NH_4)_2SO_4$ are, generally, used for this purpose.
- Precipitation of a protein by a salt solution is due to neutralization of charges as well as dehydration.
- Proteins which can be precipitated by a salt solution remain native and can, usually, be re-dissolved by putting back in the solvent.

For the **precipitation of globulins** from egg white, add **equal** volume of saturated solution of **ammonium sulfate** to the **egg white** solution. Mix and wait for 10–15 minutes. Filter the precipitate. Residue on the filter paper contains globulins.

For the **precipitation of albumin**, add ammonium sulfate powder to the filtrate to **saturate it completely**. Albumin is precipitated.

This shows that albumin is more soluble in water than globulin, since albumin precipitates at full saturation, whereas globulins precipitate at half saturation only.

By Heavy Metals

Since salts are ionic, they disrupt salt bridges in proteins. The reaction of a salt of a heavy metal with a protein usually leads to an insoluble salt of the metal with the protein. Heavy metals may also disrupt disulfide bonds because of their high affinity and attraction for the sulfur and will also lead to the denaturation of protein.

Heavy metal salts, usually, contain Hg^{2+}, Pb^{2+}, Ag^+, Cd^{2+} and other metals with high atomic weight. Most commonly used protein precipitants include metal salts such as **lead acetate**, **mercuric chloride**, **barium chloride** and **zinc sulfate**. Protein gets precipitated due to the adsorption of the metallic cations by the negatively charged colloidal particles of protein.

For the **precipitation of protein by a heavy metal**, add few drops of a salt solution of some heavy metal to 2 mL solution of egg albumin. A white precipitate indicates the precipitation of protein.

By Alkaloidal Reagents

Precipitation by an alkaloidal reagent is due to the formation of an insoluble salt between the anions of the reagent and the positively charged protein particles. This test is used for the detection of protein in urine.

To see the **precipitation of protein by an alkaloidal solution**, add few drops of an alkaloidal reagent such as **sulfosalicylic acid**, **tannic acid**, **trichloracetic acid**, **phosphomolybdic acid**, **tungstic acid** or **picric acid**, to a tube containing 2 mL solution of egg albumin. A precipitate indicates the presence of protein.

By Organic Solvents

Since proteins are, usually, soluble in water and are not soluble in organic solvents, they can be precipitated with the addition of some nonaqueous organic solvent like alcohol, acetone, etc. These are used for **protein precipitation**, often in conjunction with trichloroacetic acid.

Organic solvents decrease dielectric constant resulting in loss of solubility, due to the dehydration of the molecule, and therefore **precipitation**.

For the **precipitation of protein by an organic solvent**, add 5 mL of **alcohol** to 3 mL of egg albumin solution. A white precipitate indicates that protein has been precipitated.

COLOR REACTIONS OF PROTEINS

Due to the presence of **peptide bond**, a protein **gives** a positive **biuret test**. Due to the presence of the **specific amino acids**, proteins give various test, e.g. **xanthoproteic test**, **Millon's test**, **Hopkins-Cole test**, **Sakaguchi test** and **lead acetate test**. In general, these tests are referred to as color reactions of proteins.

Biuret Test

Peptide bonds (–CO–NH–), which are present in the protein, are responsible for the biuret test. A minimum of two consecutive peptide linkages give a positive test, i.e. minimum of a tripeptide (having two peptide linkages) is essential for the test.

This test is called biuret test because biuret, which is formed on heating urea, also gives the positive test.

Xanthoproteic Test

This reaction is due to the presence of the benzene ring, which gets nitrated in the presence of concentrated nitric acid and forms nitrobenzene derivatives, which are yellow in color.

Among the 20 amino acids, which occur in most of the proteins, only 3 amino acids, i.e. tyrosine, phenylalanine and tryptophan are **aromatic amino acids**.

Millon's Test

Tyrosine, due to the presence of the phenolic group, gives positive test with Millon's reagent.

Hopkins-Cole Test

Indole derivatives, such as **tryptophan**, give colored condensation products when treated with an aldehyde, like glyoxylic acid, in the presence of H_2SO_4 or HCl.

Sakaguchi Test

Sakaguchi test is observed due to the presence of arginine, amino acid containing a guanidino group.

Lead Acetate Test

Formation of PbS with the addition of lead acetate indicates the presence of sulfur-containing amino acids.

DENATURATION OF PROTEINS

Due to their organized structure, proteins are, usually, soluble in water. On heating or by the addition of a strong acid, protein looses its native structure and gets precipitated. This is referred to as **denaturation**.

- Denaturation of a protein results in loss of its biological activity, due to unfolding and disorganization of its secondary and tertiary structures.
- Under ideal conditions, denaturation may be reversible, however, most proteins, once denatured, remain permanently disordered.
- Denatured proteins are often insoluble and therefore can be removed from the solution.
- Denaturing agents include mechanical force, detergents, strong acids or bases, heavy metals (such as lead and mercury), alkaloidal reagents and heat.

Denaturation by Heavy Metals

Proteins can also be precipitated by denaturation by the addition of the salts of heavy metals such as lead acetate, mercuric chloride, barium chloride, zinc sulfate, etc. This is due to the adsorption of the metallic cations by the negatively charged colloidal protein particles.

Denaturation by Alkaloidal Reagents

Proteins can be precipitated by the addition of some alkaloidal reagent, e.g. picric acid. Precipitation by an alkaloidal reagent is due to the formation of an insoluble salt by the anions of the reagent and the positively charged protein particles.

Denaturation by Heat

Proteins can also be denatured by the heat. This process is referred to as **heat coagulation**. Coagulation is maximum at isoelectric pH (pI).

Important Questions

1. What are proteins? Classify them.
2. What are amino acids? Classify them.
3. Discuss briefly:
    i. α-Helix
    ii. β-Pleated sheet structure
    iii. Quaternary structure of proteins
    iv. Biological significance of amino acids
    v. Aromatic amino acids
    vi. Sulfur-containing amino acids
    vii. Essential amino acids
    viii. Branched chain amino acids
    ix. Basic amino acids
    x. Classification of amino acids according to nutritional requirement
4. Write notes on:
    i. Fibrous proteins
    ii. Globular proteins
    iii. Protein denaturation
    iv. Zwitterion

Chapter 5

Digestion and Absorption of Carbohydrates, Lipids and Proteins

DIGESTION AND ABSORPTION OF CARBOHYDRATES

DIGESTION OF CARBOHYDRATES

- Digestion of carbohydrates begins in the **mouth** where **salivary amylase** hydrolyses starch into smaller polysaccharides and maltose.
- Chewing stimulates production of saliva and mixes the enzyme (salivary amylase) with food.
- Only about 5% of the starch is hydrolyzed in the mouth. After food enters the stomach, low pH in the stomach stops the action of salivary amylase.
- After dietary contents enter **small intestine**, pancreas secretes **pancreatic amylase** and releases it through the pancreatic duct into the small intestine.
- Pancreatic amylase further continues digestion of starch and hydrolyses it to maltose, isomaltose, dextrins and branched or unbranched oligosaccharides.
- **Brush border disaccharidases** hydrolyze various disaccharides into monosaccharides (Table 5.1).

Lactose Intolerance

Lactase, present in the brush border of the enterocytes, hydrolyses lactose to glucose and galactose. **Deficiency of lactase** leads to lactose intolerance. In this condition, person complaints of abdominal pain, diarrhea and flatulence, after consuming dairy products. This is due to the reason that as lactose, due to lactase deficiency, is not digested and bacteria act upon these undigested products, leading on to various symptoms. Treatment is to withdraw milk and milk products from the diet. Curd is beneficial as it contains lactobacilli, which is a source of lactase.

ABSORPTION OF CARBOHYDRATES

As mentioned above, all the dietary carbohydrates are absorbed into the mucosal cell lining the small intestine, as monosaccharides, by two mechanisms:

- Fructose is absorbed by **facilitated diffusion**.
- Glucose and galactose are absorbed by secondary **active transport**, by the **sodium-linked glucose transporter-1** (SLGT-1).

Table 5.1 Role of various enzymes in the digestion of carbohydrates

Enzyme	Site of action	Catalytic action
Salivary amylase	Mouth	Starch/glycogen → Partially hydrolyzed dextrins, oligosaccharides, isomaltose and maltose
Pancreatic amylase	Small intestine	Partially hydrolyzed dextrins/oligosaccharides → Dextrins, maltose, isomaltose, maltotriose
α-Dextrinase	Small intestine	α-Limit dextrins → Maltotriose, glucose
Disaccharidases	Small intestine	
Sucrase		Sucrose → Glucose and fructose
Lactase		Lactose → Glucose and galactose
Maltase		Maltose/maltotriose → Glucose
Isomaltase		Isomaltose → Glucose

All the absorbed monosaccharides pass through the intestinal mucosal cells in the villi and enter liver via portal vein. Within the liver, galactose and fructose are also converted to glucose.

DIGESTION AND ABSORPTION OF LIPIDS

DIGESTION OF TRIACYLGLYCEROLS AND PHOSPHOLIPIDS

- Digestion of **triglycerides** is initiated in the **mouth with chewing** and by the action of the **lingual lipase**.
- Thereafter, with the action of **gastric lipase** and **churning and contraction** by the **stomach**, nearly 30% of the dietary triglycerides are degraded to diglycerides and free fatty acids within 2 to 3 hours.
- Thereafter, contents from the stomach pass into the **small intestine**.
- In the small intestine, it stimulates the release of two hormones, i.e. **cholecystokinin** (CCK) and **secretin**, from the duodenal cells.
- **CCK** signals gallbladder to contract and release **bile**, down the bile duct and **into the duodenum**. Bile contains large quantity of **bile salts** and **lecithin**.
- **Secretin** signals the pancreas to release **pancreatic juice** which is rich in **pancreatic lipase**.
- **Phospholipases** hydrolyse phospholipids and release monoglycerides, free fatty acids, phosphate and the nitrogenous substance (Table 5.2).

Role of Bile Salts

Bile salts help in the digestion and absorption of fats.

- **During digestion, bile salts helps in emulsification of fat:** Bile salts emulsify fat and break fat globules (clumps of triglycerides) into small pieces and keep them suspended in solution. This, in turn, increases total surface area for the action of **pancreatic lipase**. Pancreatic lipase, in turn, hydrolyzes all the accessible triglycerides, within minutes, into monoglycerides and free fatty acids.
- **In absorption, bile salts help in micelles formation:** Bile salts surround the hydrolyzed products of fat digestion and form water soluble globules with a fatty core, called **micelles**. Micelles transport monoglycerides and free fatty acids through the watery intestinal environment to the brush border of the intestinal mucosal cells for absorption.

ABSORPTION OF LIPIDS

Most of the dietary fat is absorbed **in the duodenum or jejunum of the small intestine**.

- Micelles carry monoglycerides and long chain fatty acids to the surface of the microvilli in the brush border and diffuse into the intestinal cells.
- Unabsorbed bile salts return to interior of the small intestine for the transport of monoglycerides and fatty acids.
- Bile salts are absorbed in the ileum and returned, via the portal vein, to the liver where they are again secreted into bile.
- This recycling pathway of the bile, i.e. from liver to the intestine and from intestine to the liver, is called **enterohepatic circulation**.
- After monoglycerides and fatty acids pass into the intestinal cells, they reform triglycerides.
- Most of the triglycerides, cholesterol and phospholipids combine with apoproteins and form **chylomicrons**.
- Chylomicrons make their way to the central lacteal of the villi, where they enter **lymph**, propelled through the thoracic duct and emptied into vein in the neck.

Table 5.2 Role of various enzymes in the digestion of triacylglycerols and phospholipids

Enzyme	Site of action	Catalytic action
Lingual lipase (important in neonates)	Mouth	Triglycerides → Fatty acids, monoglycerides, glycerol
Lipase/colipase	Pancreas	Triglycerides → Fatty acids, monoglycerides, glycerol
Phospholipase A/B	Pancreas	Lecithin → Fatty acid, lysolecithin
Cholesterol esterase	Pancreas	Cholesterol esters → Cholesterol, fatty acids
Retinyl ester hydrolase	Pancreas	Retinyl esters → Retinol, fatty acids
Monoglyceride lipase	Small intestine	Monoglycerides → Glycerol, fatty acids
Lecithinase	Small intestine	Lecithin → Fatty acids, glycerol, phosphoric acid, choline

- Glycerol, and medium and short chain fatty acids are absorbed directly into the blood stream (Fig. 5.1).

Dietary fat begins to appear in the blood stream after 1 to 2 hrs of meal, with a peak after 3 to 5 hrs and is generally cleared within 10 hrs. Absorption of fat is drastically reduced in the absence of bile salts. As a result of it, bulk of the unabsorbed fat is excreted. Production of stool with abnormally high amount of fat is referred to as **steatorrhea**.

DIGESTION AND ABSORPTION OF PROTEINS

DIGESTION OF PROTEINS

Digestion of proteins begins in the gastrointestinal tract and is carried out by the proteases that are secreted by the stomach, pancreas and small intestine:

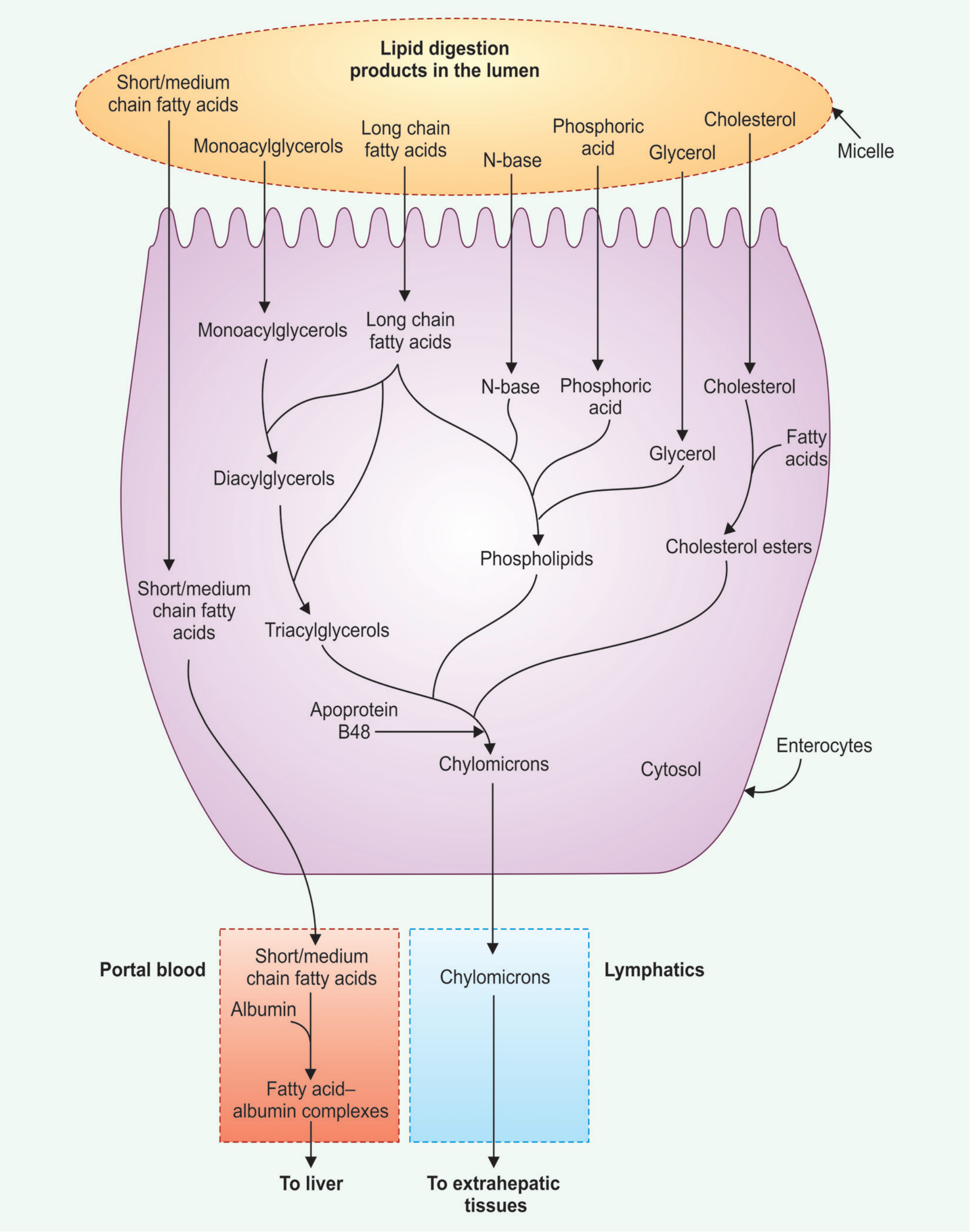

Lipid digestion products in the lumen
Short/medium chain fatty acids
Monoacylglycerols
Long chain fatty acids
N-base
Phosphoric acid
Glycerol
Cholesterol
Micelle
Monoacylglycerols
Long chain fatty acids
N-base
Phosphoric acid
Cholesterol
Fatty acids
Glycerol
Diacylglycerols
Phospholipids
Cholesterol esters
Short/medium chain fatty acids
Triacylglycerols
Apoprotein B48
Chylomicrons
Cytosol
Enterocytes
Portal blood
Short/medium chain fatty acids
Albumin
Fatty acid–albumin complexes
Chylomicrons
Lymphatics
To liver
To extrahepatic tissues

Fig. 5.1: Process of absorption of the digestion products of triacylglycerols and phospholipids

- Digestion of proteins starts **in the stomach** by the action of proteolytic enzyme **pepsin**, at pH of the gastric juice (1.5–2.5).
  Pepsin is secreted by the cells of the gastric mucosa in the form of zymogen (proenzyme), called **pepsinogen**, which is activated by **hydrogen ions** (HCl) of the gastric juice and later, autocatalytically, by pepsin itself.
- Proteolytic products of pepsin pass on to the **small intestine**, where they are attacked by the proteases, which are secreted by the pancreas. These include enzymes trypsin, chymotrypsin and carboxypeptidases.
  - **Trypsin** is secreted in the zymogen form as **trypsinogen**, which is converted to trypsin by another enzyme, called **enterokinase (enteropeptidase)**.
    Enterokinase acts as **activator of trypsinogen**. It is secreted by the intestinal mucosa. Once trypsin is formed, it acts on trypsinogen and converts it to trypsin, autocatalytically.
  - **Chymotrypsin** is also synthesized and secreted as a zymogen designated as **chymotrypsinogen**. It is activated by trypsin.
  - Pancreatic juice also contains **collagenase** and **elastase**, which specifically act on collagen and elastin, respectively.
- In addition, to the above enzymes, which are referred to as **endopeptidases**, pancreatic juice also secretes some **exopeptidases**. These are also secreted as zymogens and include **pro-carboxypeptidase A** and **pro-carboxypeptidase B**.
  - Both the pro-carboxypeptidases are **activated by trypsin**.
  - They attack **peptide linkages**, specifically those **having a free carboxyl group**.
  - **Carboxypeptidase A** hydrolyzes C-terminal amino acid, particularly, where the terminal amino acid is aromatic or acidic.
  - **Carboxypeptidase B** hydrolyzes the terminal peptide bond having a basic amino acid (arginine or lysine).
- Thereafter, **aminopeptidases** and **dipeptidases**, secreted by the mucosa of the small intestine, act on the hydrolysed products of proteins.
- Aminopeptidases act on the **amino terminal end** while dipeptidases act on **dipeptides**.
- Any part of the protein which is not hydrolyzed in the small intestine, enters the large intestine and is excreted as such in the feces.

ABSORPTION OF AMINO ACIDS

End products of the digestion of proteins, i.e. amino acids, are absorbed from the small intestine into the portal blood.

- Absorption of L-amino acids is a **Na^+-dependent active transport**. It requires ATP and the specific transport proteins.
- Absorption of amino acids also requires pyridoxal phosphate (vitamin B_6).

Transport Systems Required for the Transport of Amino Acids

Several transport systems are required for the transport of different amino acids. These include:

1. A transport system for the transport of neutral amino acids having short or polar side chain, such as Ala, Ser and Thr;
2. A separate transport system for the transport of neutral amino acids with large side chain, such as an aromatic or hydrophobic side chain, i.e. Phe, Tyr, Met, Val, Leu and Ile;
3. There is a separate transport protein for the transport of imino acids, i.e. pro and hydroxyproline (HO-Pro);
4. A separate transport system for the basic amino acids, i.e. Lys, Arg and cystine (Cys-Cys);
5. There is also a separate transport system for the acidic amino acids, i.e. Asp and Glu;
6. There is also a separate transport system for β-amino acids, e.g. β-Ala and taurine;
7. In addition, there also exists a transport system for the dipeptides, e.g. peptide-1 (pep-1), glycylsarcosine, etc. These dipeptides are absorbed through the intestinal cells, where they are hydrolyzed by dipeptidases to the constituent amino acids.

Besides, in fetus and neonates, intact proteins such as immunoglobulins, are absorbed by endocytosis (pinocytosis).

Important Questions

1. Discuss the process of digestion and absorption of dietary carbohydrates.
2. How are lipids digested? Explain the process of absorption of dietary lipids.
3. Write notes on:
    - i. Digestion of carbohydrates
    - ii. Digestion of proteins
    - iii. Role of bile salts in digestion and absorption of lipids
    - iv. Absorption of amino acids

Chapter 6

Safety Measures in a Clinical Chemistry Laboratory

The nature of the organization, required for implementation of good laboratory practice in a chemical laboratory, will depend on the size of the establishment and the nature of the work carried out. However, regardless of the size, every laboratory worker should be imparted, at least, a basic training on safety with regard to health, and safety hazards of chemicals, and the hazards which may occur from the equipment and techniques, one is likely to employ. Prevention of laboratory accidents requires great care and constant vigilance. Examples of risk factors include corrosive and toxic chemicals, high voltages, high and low pressures and temperatures, and biohazards, including infective organisms and toxins.

Measures to protect against laboratory accidents, include safety training and enforcement of laboratory safety policies, safety review of experimental designs and the use of personal protective equipment.

GENERAL RULES FOR SAFETY MEASURES IN A CLINICAL BIOCHEMISTRY LABORATORY

- Work carefully and follow the given instructions.
- Use protective clothing all the time, e.g. laboratory coat and safety glasses.
- Use a hair band, to keep hair away from the face.
- Do not smoke, eat or drink in the laboratory.
- Follow the safety instructions for the handling of chemicals.
- Keep workplace clean and organized.
- Do not cause panic in case of an unwanted chemical reaction, equipment damage or injury.
- Do not expose flammable substances to open flame.
- Wear protective gloves and safety glasses while working with concentrated acid or alkali, or whenever the mixture is reactive and aggressive.
- Do not pipette harmful or evaporable substances by mouth.
- In case, a chemical comes in contact with the skin, immediately rinse the spot with water. Neutralize and, if necessary, remove the affected clothing.
- In case a chemical comes in contact with eye(s), wash with running water, for at least 15 minutes.
- Work in fume cupboard, whenever using hazardous or toxic substances, or for experiments where easily evaporable substances or chemicals are employed.
- Pour neutral or very dilute solutions down the drain but with a lot of running water. Neutralize any acid or alkali before pouring it down the drain.
- Do not leave the laboratory without permission. Before leaving the laboratory:
  - Ensure that all the workplace is neat and clean.
  - All the chemical containers must be closed and returned to the designated place.
  - Shut-off the gas and water valves.
  - Switch-off all electric equipments.
  - Verify that there is no fire hazard.
  - Wash your hands before leaving the laboratory.

Important Questions

1. Describe various safety measures which should be observed in a clinical biochemistry laboratory.
2. List various risk factors in a clinical chemistry laboratory.

Chapter

7

Storage and Handling of Laboratory Chemicals

GENERAL RULES FOR STORAGE OF CHEMICALS

1. Do not simply store all the chemicals in alphabetical order.
2. Store like-chemicals together and away from other groups of chemicals that might cause reaction, if mixed.
3. Flammable materials should be stored in an approved, dedicated, flammable materials storage cabinet or room, if the volume is large.
4. Chemicals should be stored separately from non-compatible hazardous classes.
5. Liquids should be stored in unbreakable or double-contained packaging, or the storage cabinet should have capacity to hold the contents, if the container breaks.
6. Avoid storing chemicals on the floor, even temporarily.
7. No chemicals should be stored above eye level.
8. Avoid island shelf assemblies. They should be firmly secured to the wall. Ideally, these should be wooden.
9. Provide anti-roll-off clips on all shelves. Avoid adjustable metal shelf supports and clips.
10. Store poisons in a separate cabinet.
11. All chemicals should be labelled and dated.
12. Store light-sensitive material in a cool and dark place, in amber-colored bottles or other containers which reduce or eliminate penetration of light.
13. Look for unusual conditions in chemical storage areas, such as:
    - Leaking or deteriorating containers
    - Spilled chemicals
    - Temperature extremes, too hot or cold in storage area
    - Lack of or low lighting
    - Blocked exits and doors
    - Lack of security
    - Accumulation of trash
    - Smoking or open lights
    - Blocked, broken or missing fire equipment
    - Lack of information or warning signs, such as '**No Smoking**', '**Flammable Liquids**', '**Acids**', '**Corrosives**', '**Poisons**', **Chemical Storage**', etc.

If so, any of these conditions should be corrected, immediately. Inspection of the chemical storage area, on a routine basis, will help to correct deficiencies and prevent accidents.

CLASSIFICATION OF CHEMICALS

Chemicals are grouped in different classes, such as:

CORROSIVE CHEMICALS

A corrosive substance is one that will destroy or damage other substances with which it comes in contact. Label on the containers of corrosive chemicals, generally, has a picture as shown in Fig. 7.1.

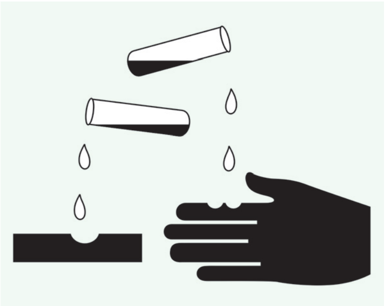


Fig. 7.1: Symbol for corrosive chemicals

Common Corrosive Chemicals

Common corrosives are strong acids, strong bases, or concentrated solutions of certain weak acids or weak bases. They can exist in any state of matter, including liquids, solids, gases, mists or vapors. Common corrosive chemicals (C) are grouped, as follows:

Acids

- **Strong acids:** Most common strong acids include sulfuric acid, nitric acid and hydrochloric acid (H_2SO_4, HNO_3 and HCl, respectively).
- Some **concentrated weak acids**, e.g. formic acid and acetic acid.
- **Strong Lewis acids**, such as anhydrous aluminum chloride and boron trifluoride.
- **Lewis acids with specific reactivity**, e.g. solutions of zinc chloride.

Bases

- Caustics or alkalis, such as sodium hydroxide (NaOH) and potassium hydroxide (KOH).
- Alkali metals such as elemental sodium, hydrides of alkali and alkaline earth metals (like sodium hydride), which function as strong bases and hydrate to give caustics.
- Extremely strong bases such as alkoxides, metal amides, e.g. sodium amide and organometallic bases, like butyllithium.
- Concentrated weak bases, such as ammonia (anhydrous or concentrated solution).
- **Dehydrating agents** such as concentrated sulfuric acid, phosphorus, calcium oxide, anhydrous zinc chloride and elemental alkali metals.
- **Strong oxidizers**, such as concentrated hydrogen peroxide.
- **Electrophilic halogens**, e.g. elemental fluorine, chlorine, bromine and iodine, and electrophilic salts, such as sodium hypochlorite or N-chloro-compounds, like chroramine-T.
- **Organic** and organic acid halides, such as acetyl chloride and benzyl chloroformate.
- **Acid anhydrides**.
- **Alkylating agents**, such as dimethyl sulfate.
- Some **organic materials**, such as phenol (carbolic acid).

Harmful Effects of the Corrosive Chemicals

- Corrosive chemicals can cause **visible destruction** or **irreversible alterations to living tissue** by chemical action at the site of contact. Their action on living tissue, e.g. skin, flesh and cornea, is mainly based on acid–base reaction of amide or ester hydrolysis.
- At low concentration, a corrosive substance is, usually, **irritant**. It may also cause **chemical burn**, on contact.
- Corrosive effects can occur not only to the skin and eyes but also to the **respiratory tract** through inhalation, and to the **gastrointestinal tract** through ingestion, which can lead to vomiting, severe stomachache and even death.
- Corrosive liquids have a high potential to cause **external injury to the body**, while corrosive gases are readily absorbed into the body through skin contact and inhalation.
- Corrosive solids and their dusts can **damage tissue** by dissolving rapidly in moisture on the skin or within the respiratory tract when inhaled.

In order to minimize these potential hazards, precautionary procedures must be observed when handling corrosive chemicals.

Handling of Corrosive Chemicals

- Appropriate personal protective equipment (e.g. gloves, fire-resistant or all cotton laboratory coat and safety goggles) must be worn when working with corrosive chemicals.
- A face shield, rubber apron and rubber booties may be appropriately used, depending upon the work performed.
- Appropriate protective gloves, which are resistant to permeation or penetration from corrosive chemicals, must be selected and tested for the absence of pin holes, prior to use.
- Eyewashes and safety showers must be readily available in the areas where corrosive chemicals are used and stored. In the event of skin or eye contact with a corrosive chemical, the affected area should be immediately flushed with water for 15 minutes.
- Contaminated clothing should be removed and medical attention sought.
- Corrosive chemicals should be handled in a fumehood to ensure that any possible hazardous or noxious fumes are adequately vented.
- When mixing concentrated acids with water, slowly add the acid to water. Allow the acid to run down the side of the container and mix slowly to avoid violent reaction and splattering. **Never add water to acid**.
- Appropriate spill clean-up material should be available in the areas where corrosive chemicals are used and stored.
- Protective carriers shall be used when transporting corrosive chemicals.

- Always handle containers of corrosive chemicals carefully, so that dusts, mists, vapors or fumes, do not get into the air. Damaged containers may leak. Acid containers, such as drums and carboys, can cause particular problems, if they are not handled and stored safely.

Storage of Corrosive Chemicals

- Containers and equipment used for storage and processing of corrosive material must be corrosion resistant.
- Corrosive chemicals must be stored below eye level, preferably near the floor, to minimize danger of their falling from the cabinet or shelf.
- Acids and caustics (i.e. bases) must be stored separately from each other. Secondary containers or trays must be used to separate acids and bases, or other incompatible corrosives, within a corrosive cabinet.
- Oxidizing acids must be separated from organic acids, as flammable.
- Combustible material (oxidizing acids) is, particularly, reactive with organics and flammable/combustible materials.
- Acids must be segregated from active metals (e.g. sodium, potassium and magnesium) and from chemicals that can generate toxic gases (e.g. sodium cyanide and iron sulfide).

TOXIC CHEMICALS

A toxic chemical or their mixture may be harmful to the human health, if inhaled, swallowed or absorbed through the skin. Label on the containers of toxic chemicals, generally, has a picture as shown in Fig. 7.2.

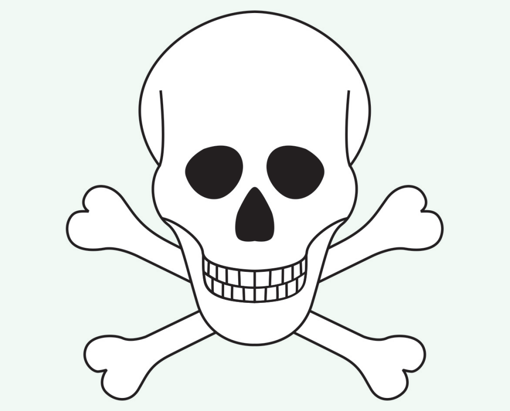


Fig. 7.2: Symbol for toxic chemicals

- These may be naturally occurring toxins (poisonous substances obtained from living organisms) found in certain plants like wild mushrooms and berries.
- Some of these may be contained in most of the everyday household products or may be synthetic (manmade).

They are divided into two groups, as **very toxic** (**T+**) and **toxic** (**T**). This classification is based on their **medial lethal dose (MLD) or LD$_{50}$** values.

MLD is the average exposure to a single dose of a chemical agent, which causes, at least, 50% of the test subjects (rats, mice, etc.) to die. There are LD$_{50}$ values for ingestion, skin exposure and inhalation of chemicals. Toxic chemicals cause damage at low levels, e.g. phosgene, hydrogen cyanide and nicotine.

Harmful Effects of Toxic Chemicals

- Some toxic materials can be harmful when in contact with **skin**. In these instances, it may be necessary to wear protective equipment such as gloves, apron, boots, hood or other preventive clothing, depending upon the risk of skin contact.
- **Eye** protection is important when working with toxic material. Potential health effects include eye irritation, toxicity through skin absorption, etc.
- Proper respiratory protection should be used.
- Ensure that emergency eyewash/shower stations are readily available nearby and are tested regularly.
- Be aware of the typical symptoms of poisoning and first-aid procedures.
- Report any sign of illness or overexposure, immediately to the supervisor. Depending on the material, medical attention for an exposure may be required even if the exposure did not seem excessive.

Handling of Toxic Chemicals

- Use only the smallest amount necessary to do the job.
- Prevent the release of toxic vapors, dusts, mists or gases into the workplace air.
- Wear appropriate personal protective equipment to avoid exposure (eye, respiratory or skin) or contact with contaminated equipment/surfaces.
- Do not return contaminated or unused material to the original container.
- Ensure containers are clearly labelled and inspect containers for leaks or damage before handling.
- To prevent spillage, use proper tools to open containers and to transfer material.
- Pour toxic liquids carefully from the container to avoid splashing and spurting.

- Avoid any welding, cutting, soldering or other hot work on an empty container or piping, until all toxic liquid and vapors have been cleared.
- Maintain good housekeeping, e.g. clean surface. There should be no accumulation of dust.

Storage of Toxic Chemicals

- Store toxins separately, away from processing and handling areas, eating area and protective equipment storage.
- The storage area should be fire-resistant and constructed with noncombustible material.
- Ensure that suitable fire extinguishers and spill clean-up equipment are available.
- Keep the amount of toxic material in storage as small as possible.
- Inspect storage area and containers regularly for any deficiencies, including leaking or damaged containers, expired shelf-life or poor housekeeping. Correct all deficiencies as soon as possible.
- Ensure that containers are tightly closed, when not in use.
- Keep empty containers in a separate storage area.
- Store containers at a convenient height for handling, below eye level, if possible. High shelving increases the risk of dropping containers and the severity of damage, injury and/or exposure, if a fall occurs.
- Store material within the temperature range recommended by the chemical manufacturer/supplier.
- To contain spills or leaks, the toxic material containers should be stored in trays made of compatible materials.

CARCINOGENS

Chemical carcinogens are substances or mixtures, which have the potential to induce cancer to human beings, under certain conditions, for prolonged period of exposure. Chemical carcinogens can be natural chemicals, synthetic compounds, or mixtures of both, that are produced or used for industrial, agricultural or commercial purposes. They are divided into 3 categories:

Category 1

It includes those substances, which are known to cause cancer in human beings. Label on the containers of carcinogens, generally, has a picture as shown in Fig. 7.3.

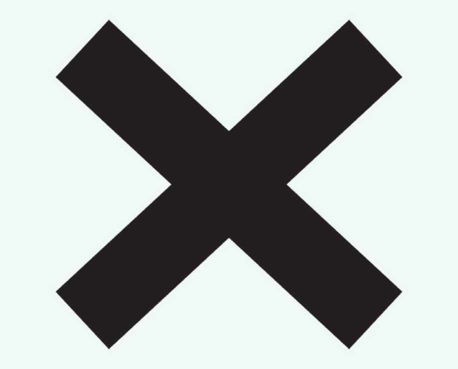


Fig. 7.3: Symbol for carcinogens

Category 2

These substances are referred to as carcinogenic to human beings, after sufficient evidence is available based on long-term animal studies and other information that their human exposure may result in cancer. These are also classified as **toxic**.

Category 3

These are the substances that may have possible carcinogenic effects. These are classified as **harmful**.

Harmful Effects of Carcinogens

- Carcinogens can cause cancer by direct action in the cellular DNA or through mechanisms that generate certain chemical species such as free radicals, reactive oxygen species, carcinogenic metabolites, etc., which enter the cell nucleus and cause mutations.
- Chemically induced cancer, generally, develops many years after the exposure to a carcinogenic agent. For example, a latency period of 30 years has been observed between the exposure to asbestos fibers (a known carcinogenic agent) and the incidence of lung cancer.
- The main routes of exposure to carcinogens come from direct contact through the methods of inhalation, injection, absorption or ingestion. Each of these exposure routes and severity of damage can vary depending on the potential carcinogen in question.

Handling of Carcinogens

- Pipetting by mouth is strictly forbidden. Mechanical pipetting aids or disposable pipetting tips should be used.
- Do not attempt to recap or cut used needles. Dispose the entire needle and syringe in a container for disposal as hazardous waste.

- Warning signs should be located on the door of the work area where carcinogens are used, e.g. caution – limited access, carcinogenic chemicals in use.
- Working surfaces should be covered with an absorbent material backed with plastic and should be replaced at regular interval or when a spillage occurs.
- All experiments involving dust, vapor or aerosols of a carcinogenic nature should be carried out in a high efficiency fume cupboard. Laminar flow cabinets do not protect the worker from exposure to the carcinogen and should not be used for this purpose.
- Under no circumstances, carcinogenic or highly toxic chemicals should be disposed of down drains or into the atmosphere.
- Personnel must wash hands and arms with soap and water, immediately after handling a carcinogen.
- Correct personal protective equipment must be worn when handling carcinogens. Ensure safety goggles, impermeable gloves, respirator, face shield, impermeable apron, closed toed shoes and long sleeve laboratory coat are worn. Disposable aprons and lab coats are preferred to reduce risk of repeated exposures.
- All surfaces should be wiped down with an appropriate cleaning agent. All waste and contaminated material must be disposed of in hazardous waste bins.
- All equipment used, must be decontaminated before removing them from the designated area.

Storage of Carcinogens

- Store in a designated area with the appropriate hazard signs and ventilation, if required.
- The carcinogens should be stored in a separate area to flammable solids and corrosive liquids, to avoid any damage to containers and possible leakage of the carcinogenic compounds.
- All carcinogenic, suspected carcinogenic or highly toxic chemicals, should be stored in screw-cap containers or ampoules, at the appropriate temperature and labelled clearly, indicating the chemical composition, date of preparation and the nature of the hazard, i.e. carcinogen–handle with care.
- Use of double containers may be necessary for certain highly potent carcinogens with physical properties that enhance spontaneous release and exposure, such as highly dispersible powders or volatile solids.

EXPLOSIVE AND FLAMMABLE CHEMICALS

Explosive chemicals are the chemicals or mixtures, which are capable of producing an explosive or pyrotechnic effect, with substantial release of heat and gases under the right conditions. Explosions can be initiated by heat, shock, friction, etc. Explosives, which are shock sensitive, are, particularly, dangerous as they can be detonated merely by touching their container. Functional groups such as azides, diazo or nitroso groups are sensitive to shock and heat, and can explode violently. Label on the containers of explosive chemicals, generally, has a picture as shown in Fig. 7.4.

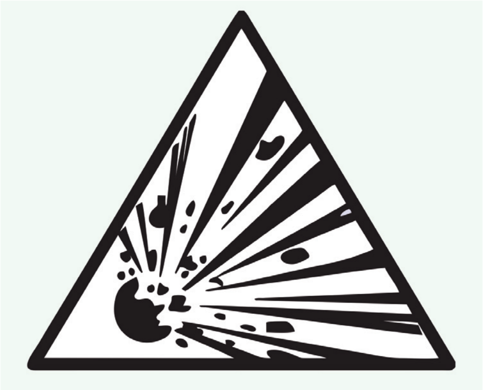


Fig. 7.4: Symbol for explosive chemicals

Flammable chemicals ignite easily. They are divided into three groups, as extremely flammable (**F+**), highly flammable (**F**) and flammable, depending upon their **flashpoint** (the lowest temperature at which the vapor on the surface of the liquid will ignite. The lower the flashpoint, easier to ignite a material). Label on the containers of flammable chemicals, generally, has a picture as shown in Fig. 7.5.

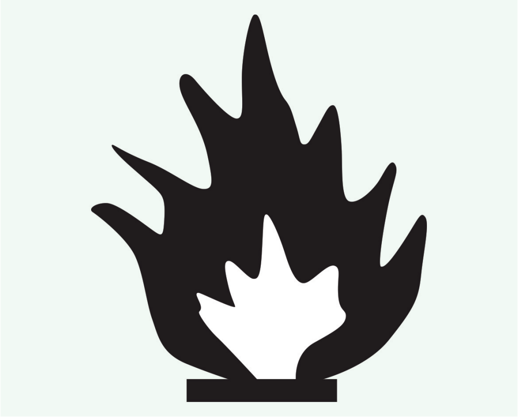


Fig. 7.5: Symbol for flammable chemicals

A majority of the organic materials are flammable. Besides, many organic solvents are highly flammable, e.g. alcohol, acetone, ether, petroleum products and toluene, besides ammonia gas, oxygen gas, methylethylketone, etc.

Harmful Effects of Explosive and Flammable Chemicals

- They are concerned with fire and explosion risks due to the potential to give rise to fire, explosion or another similar event.
- They can create thermal radiation effects (burns) and overpressure effects (blast injuries).

Handling of Explosive and Flammable Chemicals

Handling of Flammable Gases

- Staff who handle gas cylinders must be properly trained.
- Minimum number of cylinders should be used and held in laboratories.
- Cylinders should be secured to walls or benches with chains or proprietary clamps. Valves should not opened fully. Half a turn is sufficient to ensure optimum gas flow.
- All obvious ignition sources should be removed from handling areas.
- Electrical items, not in use, must be switched off and unplugged.
- Storage areas for flammable gases must be well ventilated.
- Cylinder valves should be closed immediately when no longer needed.
- Dry powder fire extinguishers must be present in the workplace.
- Skin should be protected from liquid propane, which freezes skin on contact.

Handling of Flammable Liquids

- Minimum quantities should be used, handled and stored.
- Adequate ventilation must be provided where flammables are dispensed, used or stored.
- All obvious ignition sources should be removed from the storage and handling areas. Electrical items must be safe for use in such areas.
- Nylon lab coats must not be used due to potential static problems.
- All flammable liquids must be in suitable lidded containers and stored in clearly marked bins or cupboards away from other processes and storage areas. Containers must be closed, or lid put back on, immediately when not needed and returned to the proper storage bin or cupboard.
- Glass containers of flammables must be carried carefully, so that they are not dropped or broken by striking against each other or other items.
- Dispensing from large drums to small containers should be done by trained staff. Liquids must be dispensed in a fume cupboard over spillage trays and have a stock of inert absorbent material to mop up spills.
- Solvent contaminated clothing should be removed and placed in fume cupboard immediately.
- Rags and clothes used for mopping up spills must be disposed of in metal containers with well-fitting lids, or placed in fume cupboard and removed from the workplace at the end of the day.
- Dry powder fire extinguishers should be present in the lab.

Storage of Explosive and Flammable Chemicals

- Flammable liquids must be kept in securely lidded and labelled containers.
- When moved, they should be carried in a manner to prevent breakage or spillage.
- Solvents that need to be stored at low temperatures must be kept in purpose built spark-free refrigerators, not the usual domestic type.
- Mixtures of flammable organic materials (whether gases, solvent vapors or dusts) and air may explode on ignition. To avoid this the proportion of flammable gas, vapor or dust in the air should be kept to a very low level by adopting safe working practices, giving careful thought to apparatus design, and by maintaining good ventilation, e.g. by working in a fume cupboard.
- The vicinity should be kept clear of ignition sources, e.g. flame, hot surfaces or sparks.
- Minor spillage must be cleaned up quickly. In the event of major spillage, the laboratory should be ventilated by opening the windows, evacuated and the door closed.
- Do not switch any electrical equipment on or off, since this could cause a flashback to the spillage.

Important Questions

1. Describe various rules which should be observed during the storage of chemicals.
2. What are corrosive chemicals? Describe the harmful effects of corrosive chemicals.
3. Define carcinogens. Discuss about the handling and storage of carcinogens.
4. Write notes on:
    i. Corrosive chemicals
    ii. Toxic chemicals
    iii. Carcinogens
    iv. Explosive and flammable chemicals
    v. Handling and storage of explosive and flammable chemicals

Chapter

8

First Aid and Emergency Treatment in a Clinical Chemistry Laboratory

Incidents arising as a direct result of work performed are avoidable if one observes the principle of safe working conditions. Failure to observe safe working principle results in accidents. No matter how minor is an incident, it will need proper examination and corrective measures to avoid recurrence of such incidents. Constant supervision and improvement of working methods is the best defence against the occurrence of hazards.

It is, of course, imperative that, at the earliest possible moment, all sources of danger should be eliminated. If the trouble is **electrical**, switch off the mains. If a **fire** has broken out, turn off the gas at the main, and switch off the electric power so that, if insulation is burned off from wires, an electrical fire may not start. Remove them, if there are supplies of combustible materials in the laboratory or put them away from the source of danger. **Leaking containers of chemicals**, particularly, poisonous gas or liquid should be removed to the open air, after wearing self-contained breathing apparatus and should be labelled as '**Dangerous**'. Somebody should be posted to guard and to see that no one goes near them. In any case, remove the victims to fresh air and keep them warm and at rest.

COMMON EMERGENCY PROBLEMS THAT CAN OCCUR IN A CLINICAL CHEMISTRY LABORATORY

CHEMICAL INJURIES

Chemical injuries are commonly encountered, especially following exposure to acid and alkali, anhydrous ammonia or phenol.

Skin Contact

Observe the following **general rules**, if skin is affected by chemicals:

- Flood the affected skin thoroughly with large quantities of running water for at least 10 minutes, or till satisfied that no chemical is in contact with the skin. Use of soap will facilitate the removal of chemicals insoluble in water.
- Remove all contaminated clothing of the casuality, ensuring that the person removing the clothing is not contaminated during the process.
- If necessary, arrange for transport to a hospital or to a doctor for medical attention. The information regarding the hazardous chemical along with details of first aid given should accompany the casuality.

Acid Splashes on the Skin

- Wash the affected skin with plenty of tap water. Bathe the affected skin with cotton wool soaked in 5% solution of sodium carbonate.
- Rinse the affected skin in mild detergent and then contact a physician.

Alkali Splashes on the Skin

- Wash the affected skin with plenty of tap water.
- Bathe the affected skin with cotton wool soaked in 5% acetic acid and seek medical help.

Contact with Phenol

- Wash the affected skin with large volumes of tap water.
- Use polyethylene glycol (PEG) mixed with water for further irrigation of affected skin and consult a physician.

Accidental Swallowing

Accidental swallowing of poisonous reagent

- Spit it out immediately.
- Rinse the mouth promptly with tap water.

- Induce vomiting by drinking warm salt water (one tablespoon of common salt in glass full of warm water).

Accidental swallowing of infectious specimen

- Spit it out immediately.
- Wash the mouth with dilute antiseptic lotion.
- Rinse the mouth thoroughly with tap water.

Accidental swallowing of corrosive reagents

- **Acids**
  - Promptly rinse the mouth with tap water.
  - Antidote such as 5% soap solution, 8% magnesium hydroxide or white of egg mixed with about 500 mL of water, can be used orally to neutralize the acid.
  - Seek medical help immediately.
- **Alkalies**
  - After rinsing the mouth with tap water
  - Antidote such as lemon juice or 5% acetic acid can be taken orally to neutralize the alkali.
  - Seek medical help immediately.

Contact of lip and tongue with corrosive reagents

- **Acids:** Immediately rinse with tap water and then bathe the affected part in 2% aqueous sodium carbonate.
- **Alkalies:** Immediately rinse in tap water and then bathe the affected part in 5% acetic acid.

Ingestion

- If the chemical is confined to the mouth, large quantities of water should be given as a mouthwash to the casualty.
- If the chemical has been swallowed, dilute the chemical by giving copious drinks of water or milk.
- Do not induce vomiting.
- Arrange for transport to hospital for medical attention, giving information regarding the chemical swallowed, its concentration and quantity, if possible along with details of first aid given should accompany the casualty.

Chemical Splash in the Eye (Fig. 8.1)

- Thoroughly wash the eyes with large quantities of gently running water from a tap or wash bottle for 10 minutes.
- Make sure that the water bathes the eyeball by gently opening the eyelids and keeping them apart till the treatment is complete.

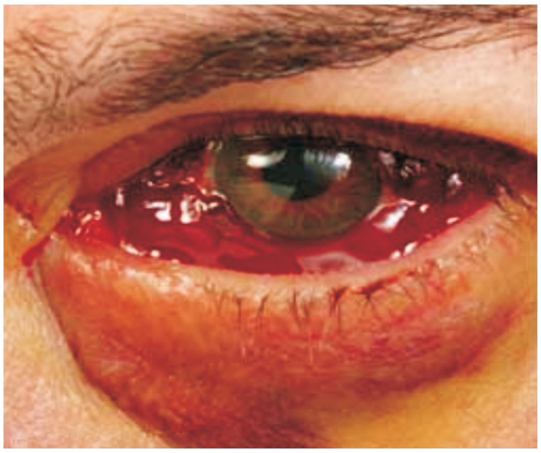


Fig. 8.1: Chemical splash in eye

- Arrange for transport to hospital for immediate medical attention and treatment of eye injury. Information regarding the chemical should accompany the patient.

Inhalation of Vapors/Gases

- Remove the affected person out of the danger area to fresh air.
- Loosen clothing.
- If the affected person is unconscious, put the person in a face down position and watch to see if breathing stops.
- If breathing has stopped artificial respiration by the mouth-to-mouth method should be applied.
- Remove the person for immediate medical aid to a hospital accompanied with information on the chemical and details of first aid given.

Chemical Burns (Fig. 8.2)

- Remove patient from the accident site.

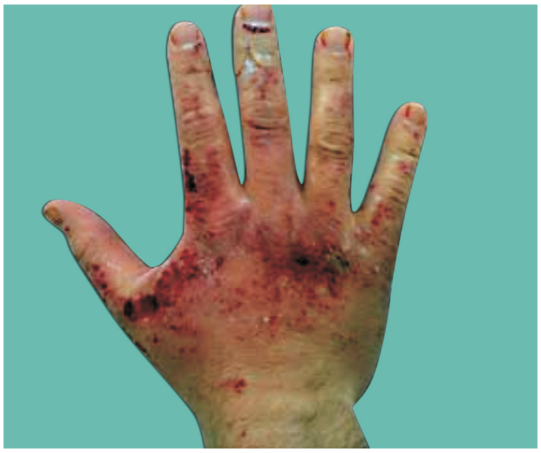


Fig. 8.2: Chemical burns

- Flood area with cool water for at least 20 minutes or until help arrives.
- Make sure water doesn't flow on to another part of the person's body or on to you.
- Do not use a strong stream of water, if possible.
- As you flush the burn (not before), remove jewellery or articles of clothing with chemical on them, unless they are stuck to the person's body.
- Identify chemical for effective therapy and follow instructions on the label of the chemical product, if available.
- Do not try to neutralize the burn with acid or alkali. This could cause a chemical reaction that worsens the burn.
- Do not put antibiotic ointment on the burn.
- Seek medical treatment immediately.

THERMAL INJURIES

Burns may be caused while working in the laboratory.

1. Minor Burns

- Immediately bathe the affected area in cold water.
- Cover the burnt area with sterile dressing.
- Seek medical help.

2. Severe Burns

- If the victim is on fire, put out the fire by using sheet, towel or coat.
- Pour lot of cold water on the affected area.
- Remove the smoldering clothes as soon as possible.
- Take the victim to casuality.

MECHANICAL INJURIES

They may seldom be caused while working in a laboratory. Depending upon the degree of injury caused, management should be done. The minor injury can be managed in the laboratory itself but for a severe injury, say some soft tissue injury or a minor fracture, the affected part should be immobilized with a splint and the person should be transferred to casuality for proper management.

ELECTRICAL INJURIES

- Most important is to turn off power. Unplug an appliance, if plug is undamaged. Alternatively, shut off power via circuit breaker, fuse box or outside switch (Fig. 8.3).
- If power cannot be turned off, try to separate the person from the current source using non-conductive object, such as wooden or plastic broom handle, chair or rubber doormat.
- Check for other injuries. If:
  - The victim is **unconscious** or has stopped breathing: Begin artificial respiration without delay. Continue until the first-aid group arrives.
  - **Broken bones or dislocation:** Keep the victim still. Do not manipulate the injury.
  - **Seizures, shock, heart attack:** If the face is white, place upper part of the body in low position. If the face is red, place upper part of the body in a high position.
  - **Convulsions/fits:** Lay the patient down, carefully, protecting his head with a pillow or clothing.

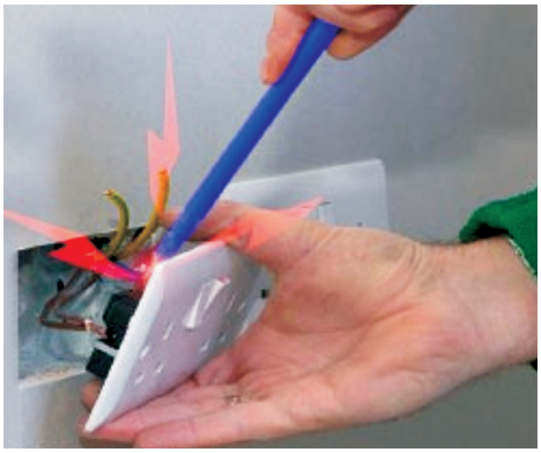


Fig. 8.3: Electric shock

Call emergency medical aid as excessive burns may require hospitalization/surgery.

INJURIES CAUSED BY BROKEN GLASS

Take the following steps for minor cuts:

1. Stop the bleeding: Apply direct pressure on the area.
2. Clean and protect: Clean the area with warm water and gentle soap or a disinfectant solution, such as dettol.
3. Apply an antibiotic ointment to reduce chance of infection. Put a sterile bandage and adhesive tape on the area.
4. Call a health care provider.

FIRST AID

First aid is the temporary help given to an injured or a sick person before professional medical treatment can be provided. The timely assistance, comprising of simple medical techniques, is most critical to the victim and is, often, life-saving. Any layperson can be trained to administer first aid, which can be carried out using minimal equipment.

First aid must be provided in any chemical laboratory. Information regarding the location of first aid and first-aider should be displayed in all places of work. All laboratories should maintain prescribed number of first-aid boxes containing copy of leaflet giving advice on first-aid treatment.

First-Aid Box

A first-aid box should contain sufficient number of sterilized, unmedicated dressings of various sizes, adhesive wound dressings, plaster, sterilized absorbent cotton, eye pads, approved eye ointment and safety pins. In addition, eye-wash bottles with clean water should be placed at strategic points.

EMERGENCY TREATMENT OF SHOCK/UNCONSCIOUS PATIENT

1. The crowding should be avoided around the patient so that he gets enough air to breathe.
2. Lay the person down and elevate the legs around 12 inches above the ground unless there is an injury in the back, head or neck or a broken hip or leg bones.
3. Begin CPR (cardiopulmonary resuscitation), if the breathing seems difficult.

CPR is an emergency procedure that combines chest compressions often with artificial ventilation in an effort to manually preserve intact brain function until further measures are taken to restore spontaneous blood circulation and breathing in a person who is in cardiac arrest.

- Give 30 chest compressions.
- Perform the head tilt, chin lift maneuver to open the airway and determine if the patient is breathing.
- Before beginning ventilations, look in the patient's mouth for a foreign body blocking the airway.
- With the airway open (using the head tilt, chin lift maneuver), pinch the nostrils shut for mouth-to-mouth breathing and cover the person's mouth with yours, making a seal.
- Resume chest compressions to restore circulation.
- Adult CPR—compressions: When performing chest compressions, proper hand placement is very important. To locate the correct hand position place two fingers at the sternum (the spot where the lower ribs meet), then put the heel of your other hand next to your fingers.
- 100 chest compressions should be given per minute. The compression depth should be two inches, that is compression should be hard and fast.
- After 30 compressions 2 breaths are given.

This emergency treatment can be life-saving for a patient and should be continued, on the patient, on the way to the hospital.

Important Questions

1. Describe common emergency problems that can occur in a clinical chemistry laboratory.
2. Write notes on:
   i. First aid in clinical chemistry lab
   ii. Emergency treatment to a patient of shock

Chapter
9

Units of Measurements

For most quantities, a unit is absolutely necessary to communicate the values of the physical quantity. For example, conveying to someone a particular length without using some sort of unit is impossible, because a length cannot be described without a reference used to make sense of the value given. Thus, performing a measurement means comparing an unknown physical or chemical quantity with a quantity of the same type taken as reference, using an instrument. A measurement necessarily involves a reference frame and therefore **unit**.

A **unit of measurement** is a definite magnitude of a physical quantity, defined and adopted by convention, or by law, and that it is used as a standard for the measurement of the same physical quantity. Any other value of the physical quantity can be expressed as a simple multiple of that unit of measurement. For example, **length is a physical quantity and that meter (metre) is a unit of length**, which represents a definite predetermined length. When we say 10 metres (10 m), we actually mean 10 times the definite predetermined length called metre.

Units of measurement were among the earliest tools invented by humans. In the past, there were **numerous units**, which had little in common with each other. These include the following.

Traditional Units

Historically, many of the systems of measurement which had been in use to some extent were, based on the **dimensions of the human body**, according to the described proportions. As a result, units of measure could vary, not only from location-to-location but also from person-to-person.

Metric Units

The **first coherent system of units** appeared with the French revolution and was referred to as the metric system. This system was internationally **ratified by the Metre Convention** in 1875, a diplomatic treaty, which had set up the Bureau International des Poids et Measures (BIPM).

Natural Units

While the above systems of units are based on arbitrary unit values, formalized as standards, some unit values occur naturally in science. Systems of units based on these values are called natural units.

Similar to natural units, **atomic units** (au) are a convenient system of units of measurement used in atomic physics.

INTERNATIONAL SYSTEM OF UNITS (SI UNITS)

This is the most widely used system of units. It was adopted in 1960, during the eleventh General Conference of Weights and Measures (Conférence Générale des Poids et Measures; CGPM), and formed the basis of the International System of Units (Systeme Internationale de Units; SI Units). These recommendations were later accepted, in principle, by the **Commission on Clinical Chemistry** of the **International Union of Pure and Applied Chemistry** (CCC-IUPAC) as well as by the **International Federation of Clinical Chemistry** (IFCC).

The international system of units is a derivative of the metric system and consist of:

- The **base units**; and
- The **derived units**.

Table 9.1 Base units of measurements

Physical quantity	Base unit	Symbol	Definition
Length	Metre	m	Metre is the length of the path travelled by light in vacuum during a time interval of 1/299,792,458 of a second.
Mass	Kilogram	kg	Kilogram is the mass of the platinum–iridium prototype, which was approved by the Conférence Générale des Poids et Measures, held in Paris in 1889, and kept by the Bureau International des Poids et Measures.
Time	Second	s	Second is the duration of 9,192,631, 770 periods of the radiation, corresponding to the transition between the two hyperfine levels of the ground state of the caesium 133 atom.
Electric current	Ampere	A	Ampere is the intensity of a constant current, which, if maintained in two straight parallel conductors of infinite length of negligible circular cross-section and placed 1 metre apart in vacuum, would produce between these conductors a force equal to 2×10^{-7} newton per metre of length.
Thermodynamic temperature	Kelvin	K	Kelvin is the fraction 1/273,16 of the thermodynamic temperature of the triple point of water.
Luminous intensity	Candela	cd	Candela is the luminous intensity, in a given direction, of a source that emits monochromatic radiation of frequency 540×10^{12} hertz and that has a radiant intensity in that direction of 1/683 watt per steradian.
Amount of substance	Mole	mol	Mole is the amount of substance of a system which contains as many elementary entities as there are atoms in 0.012 kilograms of carbon-12.

Base Units

Base units are defined in terms of the standard physical properties of selected atoms or molecules and comprise of seven dimensionally independent physical quantities, which contain no numerical factor other than unity (Table 9.1).

These base units may be used singly or in combination, to describe most quantitative measurements. These include:

- Metre (m)
- Kilogram (kg)
- Second (s)
- Ampere (A)
- Kelvin (K)
- Candela (cd)
- Mole (mol)

Derived Units

There are numerous derived units that are obtained from the base units, according to algebraic relations, using mathematic operations linking corresponding quantities.

There are two types of the derived units:

Derived Coherent Units

These are constructed from the base units only (Table 9.2).

These are complementary to the base units. They may have special names (e.g. hertz, pascal, etc.) but can always be expressed in terms of the base units.

Table 9.2 The derived coherent units of measurements

Measurement	Derived unit	Symbol
Current intensity	Ampere	A
Electrical resistance	Ohm	Ω
Power	Watt	W
Energy	Joule	J
Mass	Kilogram	kg
Density	—	Kg/m^3
Volume	—	m^3
Force	Newton	N
Pressure	Pascal	Pa
Length	Metre	m
Wavelength	Metre	m
Temperature	Kelvin	K or °C
Amount of substance	Mole	mol
Ionizing radiation	Emission rate	Bq or Ci
Frequency	Hertz	Hz
Time interval	Second	s

These units are linked together to form a coherent system.

Dimensionless Derived Units

These units are linked together to form a consistent system.

Each quantity needs to cover a vast range of values.

Prefixes

To avoid the need for multiplying factors or values with a large number of zeros, a **prefix** is used with the unit. The prefixes cover a range extending from 10^{12} to 10^{-12} times the units (Table 9.3).

Table 9.3 The prefixes used with the units of measurements

Factor	Prefix	Symbol
10^{12}	Tera	T
10^{9}	Giga	G
10^{6}	Mega	M
10^{3}	Kilo	k
10^{2}	Hecto	h
10^{1}	Deka	da
10^{-1}	Deci	d
10^{-2}	Centi	c
10^{-3}	Milli	m
10^{-6}	Micro	µ
10^{-9}	Nano	n
10^{-12}	Pico	p

Some basic units are still used in conjunction with the SI units (Table 9.4).

Various prefixes in use with these units are shown in Table 9.5.

Table 9.4 Basic units in use with SI units

Physical quantity	Name of SI unit	Symbol
Length	Metre	m
Mass	Kilogram	Kg
Time	Second	s
Electrical current	Ampere	A
Amount of substance	Mole	mol
Thermodynamic temperature	Kelvin	K
Force	Newton	N
Pressure	Pascal	Pa
Power	Watt	W
Electrical charge	Coulomb	C
Electric potential difference	Volt	V
Electrical resistance	Ohm	Ω
Frequency	Hertz	Hz
Area	Square metre	m^2
Volume	Cubic metre	m^3
Density	Kilogram per cubic metre	kg/m^3
Electric field strength	Volt per metre	V/m
Concentration	Mole per cubic metre	mol/m^3

Table 9.5 Prefixes and their symbols used with the SI units

Prefix	Symbol	Factor	Numerical value
Mega	M	10^{6}	10,00,000
Kilo	k	10^{3}	1000
Hecto	h	10^{2}	100
Deka	da	10^{1}	10
Deci	d	10^{-1}	0.1
Centi	c	10^{-2}	0.01
Milli	m	10^{-3}	0.001
Micro	µ	10^{-6}	0.000,001
Nano	n	10^{-9}	0.000,000,001
Pico	p	10^{-12}	0.000,000,000,001

1. Gram (g)

Gram (g) is used as an elementary unit in association with mg and pg.

- 1 kg = 1000 g
- 1 g = 1000 mg
- 1 mg = 1000 µg

2. Litre (L)

Although SI unit for volume is cubic metre (m^3), litre (L) is accepted as the convenient unit of volume.

- 1 litre = 1000 mL
- 1 mL = 1000 µL

3. Minute

Although the basic unit for time is second, common units used for time are minute and hour. Thus, some non-SI units are also in common use (Table 9.6).

Table 9.6 Non-SI units in common use

Quantity	Unit	Symbol	Value in S units
Time	Minute	min	1 min = 60 s
	Hour	h	1 h = 3600 s
	Day	d	1 d = 86400 s
Volume	Litre	L	1 L = 1000 mL

4. Mole (mol)

The basic SI unit of quantity is mole. It is the amount of a substance present in a container (irrespective of the volume present). It is defined as the molecular weight of a compound in grams. For example, as molecular weight of sodium chloride is 58.5, i.e. 1 mole of sodium chloride is 58.5 g. Similarly, 1 mole of glucose (mol. wt. 180) is 180 g.

$$\text{Mole} = \frac{\text{Mass (g)}}{\text{Gram molecular weight}}$$

$$\text{Molarity of a solution} = \frac{\text{Number of moles of solute}}{\text{Number of litres of solution}}$$

A molar solution contains 1 mole of solute in 1 L of solution (1 mol/L)

- 1 mol/L = 1000 millimoles/L
- 1 millimol/L = 1000 micromoles/L

5. Mass

$$\text{Mass} = \frac{\text{Mass of a component}}{\text{Mass of system (mixture)}}$$

For example, 10 g/dL (w/v) sodium hydroxide means 10 g of sodium hydroxide dissolved in 100 mL of distilled water or 100 g sodium hydroxide dissolved in 1 L of distilled water.

6. Gas Concentration and Pressure

The common units for gas concentrations are:

- volume per cent (v/v), or
- mmol/L

The common unit of expression for partial pressure of a gas is mmHg.

7. Electrolyte Concentration

The common unit for electrolyte concentration is milliequivalents/litre (mEq/L).

$$\text{mEq/L} = \frac{\text{mg/dL} \times 10}{\text{Equivalent weight}}$$

8. Ionic Strength

The effect of a buffer solution is a function of the concentration and the charge of all ions present. Hence, concentration of a buffer solution is given in terms of ionic strength. Ionic strength is the sum of the numbers obtained by multiplying the concentration (moles/litre) of each ion by the square of its valency and dividing the sum by 2. For example:

a. 0.5 mole/L NaCl

$$\text{Ionic strength} = \frac{0.5 \times (1)^2 + (0.5) \times (1)^2}{2} = 0.5$$

b. 0.2 mole/L Na_2SO_4

$$\text{Ionic strength} = \frac{0.2 \times 2 \times (1)^2 + 0.2 \times (2)^2}{2} = 0.8$$

$$\text{mmol/L} = \frac{\%\ \text{concentration} \times 10}{\text{Molecular weight}}$$

9. Heat

$$C = \frac{5 \times (F - 32)}{9}$$

where C = Celsius (°C) and F = Fahrenheit (°F)

Important Question

1. Write short notes on:
   - i. Base SI units
   - ii. Derived units
   - iii. SI units
   - iv. Basic units in use with SI units in clinical chemistry

Chapter

10

Radioisotopes and Radiation Hazards

The term isotope is derived from the Greek words "*iso*, means **equal**, and *topos*, means **place**, i.e. meaning thereby "the same place". Accordingly, different isotopes of a single element occupy the same position on the periodic table.

- The number of **protons** within the atom's nucleus is called **atomic number**, which is **equal to the number of electrons** in the neutral (nonionized) atom.
- **Isotopes** are the variants of a particular chemical element, which **differ in the number of neutrons** although all the isotopes of a given element have the **same number of protons** in each atom.
- Thus, **isotopes** of an element have the same number of protons, i.e. **same atomic number** but different masses due to **different number of neutrons**.

  An atom of a given element may, thus, have a wide range in its number of neutrons.
- The number of nucleons, i.e. both protons and neutrons, in the nucleus is the atom's mass number, and each isotope of a given element has a different **mass number**. For example, carbon-12, carbon-13 and carbon-14 are the three isotopes of the element carbon with mass numbers 12, 13 and 14, respectively. As we know, the atomic number of carbon is 6, which means that every carbon atom has 6 protons, so the neutron numbers of these isotopes are 6, 7 and 8, respectively.
- Some isotopes do not undergo radioactive decay and are described as **stable isotopes** or **stable nuclides**.
- On the other hand, there are some isotopes which undergo radioactive decay and are described as **radioisotopes** or **radionuclides**.

RADIOISOTOPES

When a combination of protons and neutrons is produced artificially, the resulting unstable atom is called a radioisotope.

The nucleus of a radioisotope becomes stable by emitting α or β (positron) particles, with or without γ radiation (electromagnetic waves). This is called **radioactive decay**.

Spontaneous decay of radioactive materials produces radiation.

- Radiation may be **ionizing** or **nonionizing**.
- **Alpha**, **beta**, **gamma** and **X-ray** particles are the most common forms of **ionizing radiations**.
- The **amount of energy**, the radiation can deposit in a given space, **varies with each type**.
- Radiations also **differ in the power to penetrate**.
- Inside the body, the **alpha particle** (**2 protons + 2 neutrons**) will deposit all its energy in a very small volume of tissue, thus they are most damaging to the tissues while **gamma-radiation** (electromagnetic waves with maximum penetration power) will spread energy over a much larger volume. **X-rays** are also electromagnetic waves but less powerful than gamma rays.
- Radioactive **iodine is a beta particle** (**one electron**), released during nuclear plant accidents.

HALF LIFE OF RADIOACTIVE ISOTOPES

The half life is the time taken for a radioactive isotope to become half of its original activity. It is constant for a particular isotope. A decay time of 7 half lives reduces the radioactivity to less than 1%. This is of importance in planning experiments and disposal of radioactive waste.

USE OF RADIOISOTOPES IN MEDICINE

There are nearly one hundred radioisotopes whose beta and/or gamma radiation is used in diagnosis, therapy or investigations, in nuclear medicine. Some of the well-known radioisotopes are ^{131}I, ^{60}Co, ^{99m}Tc and ^{137}Cs.

Diagnostic procedures using radioisotopes are also being used in routine in **nuclear medicine** to provide diagnostic information about the functioning of a person's specific organs or to treat them.

Nuclear medicine is a branch of medicine, which employs **radiation** as a diagnostic and therapeutic tool. Radioactive products used in nuclear medicine are referred to as **radionuclides** or pharmaceuticals labeled with radionuclides, called **radiopharmaceuticals**.

It is used to treat some medical conditions, especially cancer, using radiation to weaken or destroy particular targeted cells.

As Diagnostic Tools in Nuclear Medicine

As we know, every organ in our body acts differently from a chemical point of view. Hence, a number of chemicals have been identified which are absorbed by specific organs. For example, the thyroid takes up iodine, the brain consumes a large quantity of glucose, and so on. With this knowledge, radioisotopes are attached to biologically active substances and once a radioactive form of one of these substances enters the body, it is incorporated into normal biological processes and are excreted in the usual way.

- A radioisotope used for diagnosis must emit gamma rays of sufficient energy to escape from the body and it must have a half life short enough for it to decay away, soon after imaging is complete.
- Radionuclides or radiopharmaceuticals are administered to patients and the radiation emitted is measured, generating an image, with a gamma camera. This technique is referred to as **imaging/ scintigraphy**.
- It is useful to assess:
  - **Blood flow to an organ**, e.g. thalium-201 for myocardial perfusion scan.
  - **Organ function**, e.g. iodine-131 for thyroid.
  - **Presence of cancer**, e.g. fluorine-18 labeled deoxyglucose. It is readily incorporated into the cell without being broken down and is a good indicator of cell metabolism.
  - **Presence of infection**, e.g. technetium-99m labeled ciprofloxacin.
  - **Response to treatment**.
  - **Myocardial perfusion imaging** (MPI) uses **thallium-201** chloride or technetium-99m and is important for the detection and prognosis of coronary artery disease.

As Therapeutic Tools in Radiotherapy

Rapidly dividing cells, such as cancer cells, are very sensitive to radiation damage. Thus, radionuclide therapy (RNT) or simply **radiotherapy** is employed to target some cancerous growths. These include:

- **External irradiation** (external RNT, sometimes called **teletherapy**) is carried out using a gamma beam (γ-rays) from a radioactive cobalt-60 source. An external radiation procedure is known as the gamma knife radiosurgery and involves focussing gamma radiation on a precise area of the brain with a cancerous tumor.
- **Internal radionuclide therapy** is by administering or planting a small radiation source, usually a gamma or beta emitter (γ or β emitter) in the body, usually close to the cancer cells. This may be a short range or targeted therapy.
  - **Short range radiotherapy (brachytherapy):** This is becoming the main means of treatment. Iodine-131 is commonly used to treat thyroid cancer. It is also used to treat nonmalignant thyroid disorders. Iridium-192 implants are used especially in the head and breast.
  - **Targeted alpha therapy (TAT) or alpha radioimmunotherapy:** This is used for dispersed cancers where tissue specific monoclonal antibodies, attached to a emitters (e.g. bismuth-213), are used as carriers.

Side Effects

1. Some radioisotopes have carcinogenic potential. It thus, becomes an occupational hazard. Care should be taken to follow the safeguard rules, stringently.
2. Radiations given during cancer treatment also produce lot of free radicals in tissues, which have a catastrophic effect on different biological compounds. Effect on various tissues like GIT, skin, hair, mucous membrane, blood cells and reproductive organs almost always accompany the treatment protocol, in spite of all the protections used during the radiation process.

In Research

Isotopes of an element will have identical chemical reactions. Hence, when a radiolabeled compound is administered, these molecules are metabolized by the body similar to normal molecules. This is called tracer technique. Almost all biochemical research uses such tracer methods.

In Sterilization of Hospital Supplies

Gamma irradiation is widely used for sterilizing medical products and supplies, such as syringes, gloves, clothings and instruments, etc., many of which get damaged by heat sterilization. Cobalt-60 is the main isotope used, since it is an energetic gamma emitter. Small gamma irradiators, often with Cs-137, are used for treating blood for transfusion and for other medical applications.

USE OF RADIOISOTOPES IN BIOCHEMISTRY

It is very easy to detect the presence or absence of some radioactive material even when it exists in very low concentration. Therefore, several radioisotope-labeled compounds are used to label the molecules of biological samples *in vitro*. Thus:

- Radioisotopes help in elucidating the origin and fate of various biomolecules in the body as well as in the understanding of metabolic pathways in radioisotope tracer studies.
- A large number of tests have been devised to determine the constituents of blood, serum or urine, hormones, antigens and many drugs, by means of the associated radioisotopes. These procedures are known as **radioimmunoassays** (RIA). The RIA kits, manufactured for laboratory use, are very easy to use and give accurate results (Chapter 50).

COMMONLY USED RADIOISOTOPES IN MEDICINE

- **Calcium-47:** Important aid to biomedical researchers, studying the cellular functions and bone formation in mammals.
- **Carbon-14:** Major research tool used in biological research.
- **Cesium-137:** Used to treat cancerous tumors.
- **Chromium-51:** Used in research in red blood cell survival studies.
- **Cobalt-57:** Used as a tracer to diagnose pernicious anemia.
- **Cobalt-60:** Used to sterilize surgical instruments. Also used in cancer treatment and radiography.
- **Copper-67:** When injected with monoclonal antibodies into a cancer patient, helps the antibodies bind to and destroy the tumor.
- **Gallium-67:** Used in medical diagnosis.
- **Iodine-123:** Widely used to diagnose thyroid disorders and other metabolic disorders, including brain function.
- **Iodine-125:** Major diagnostic tool used in clinical tests and to diagnose thyroid disorders. Also used in biomedical research.
- **Iodine-129:** Used to check some radioactivity counters in *in vitro* diagnostic testing laboratories.
- **Iodine-131:** Used to treat thyroid disorders (Graves disease).
- **Phosphorus-32:** Used in molecular biology and genetics research.
- **Strontium-85:** Used to study bone formation and metabolism.
- **Technetium-99m:** Most widely used radioactive pharmaceutical for diagnostic studies in nuclear medicine. Its different chemical forms are used for brain, bone, liver, spleen and kidney imaging, and also for blood flow studies.
- **Thallium-201:** Used in nuclear medicine for nuclear cardiology and tumor detection.
- **Xenon-133:** Used in nuclear medicine for lung ventilation and blood flow studies.

RADIATION HAZARDS

As mentioned above, radioactive substances emit continuously powerful radiations, such as α, β and γ particle rays. Harmful effects on an organism, caused by these radiations, are called **radiation hazard**. They damage cells and tissues, which in turn, hamper normal functioning of the living system and may lead to death. M. Curie and E. Fermi, lost their lives because of the damage caused by these radiations.

Radiation protection is defined as the protection of people from harmful effects of exposure to ionizing radiation and the means for achieving this. Fundamental to radiation protection is the reduction of expected dose and measurement of human dose uptake. There are three factors that control the amount or dose of radiation, received from a source. Radiation exposure can be managed by a combination of these factors, i.e.:

1. **Time:** Reducing the time of exposure reduces the effective dose, proportionally. An example of reducing radiation doses by reducing the time of exposure is by improving operator's training to reduce the time he takes to handle a source.
2. **Distance:** Increasing distance, reduces dose. Distance can be as simple as handling a source with the forceps than directly by fingers.
3. **Shielding:** The term biological shield refers to a mass of absorbing material placed around a reactor or other radioactive source, to reduce the radiation to a level that is safe for human.

Safety Precautions from Radiation Hazards

1. The radioisotopes should be transferred in thick wall lead containers and should be kept in rooms with thick walls of lead.

2. Radioisotopes should be handled with the help of remote control device.
3. Workers must be asked to wear lead aprons.
4. Radioactive contamination of the working area should be avoided as far as possible.

Important Questions

1. Define radioisotopes. Describe the use of radioisotopes in medicine.
2. Write notes on:
   - i. Isotopes
   - ii. Radioisotopes
   - iii. Half life of radioactive isotopes
   - iv. Commonly used radioisotopes in medicine
   - v. Use of radioisotopes in biochemistry

Chapter

11

Biophysical Chemistry

Biophysical chemistry refers to certain physio-physical phenomena, which occur in the biological systems, such as osmosis, dialysis, surface tension, etc.

OSMOSIS

Osmosis refers to the spontaneous **net movement of solvent molecules through a partially permeable membrane** into a region of higher solute concentration, in the direction that tends to equalize the solute concentration on the two sides (Fig. 11.1).

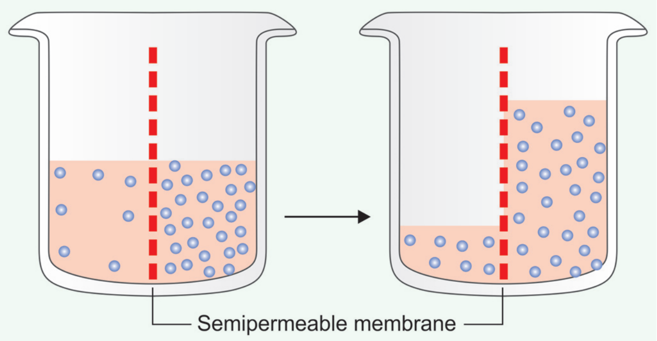


Fig. 11.1: Osmosis

- Osmosis may be described as a **physical process in which any solvent moves across a semipermeable membrane**, i.e. the membrane which is permeable to the solvent but not the solute, **separating two solutions of different concentrations**.
- It is a vital process in biological systems as biological membranes are semipermeable.
- When a cell is submerged in water, the water molecules pass through the cell membrane from an area of low solute concentration to high solute concentration. For example, if an animal cell is placed in a solution of sugar or salt in water:
  – **If the medium is hypotonic** relative to the cell cytoplasm, the cell will gain water through osmosis. This means that the **cell will swell** and may even burst.
  – **If the medium is isotonic** there will be **no net movement of water** across the cell membrane.
  – **If the medium is hypertonic** relative to the cell cytoplasm, the **cell will lose water by osmosis**. Essentially, this means that it will shrivel.

Osmotic Pressure

Osmotic pressure is defined as the **pressure exerted by the flow of water through a semipermeable membrane**, separating two solutions with different concentrations of solute. It needs to be applied to a solution to prevent the inward flow of water across a semipermeable membrane.

This process is of vital importance in biology as the cell's membrane is selective towards many of the solutes found in living organisms.

In order to visualize the effect:

- Imagine a U-shaped tube with equal amounts of water on each side, separated by a water permeable membrane, made from dialysis tubing at its base, which is impermeable to sugar molecules.
- After the sugar has been added to the water on one side, the height of the liquid column on that side will rise (and that on the other side will drop) proportional to the pressure of the two solutions, due to movement of the pure water from the compartment without sugar into the compartment containing sugar.
- This process will stop once the pressure on both sides of the membrane becomes equal.

The osmotic pressure (Π) of an ideal solution with low concentration can be approximated using the

Morse equation:

$$\Pi = iMRT,$$

where i = Dimensionless van't Hoff factor

M = Molarity

R = 0.08205746 L atm K^{-1} mol^{-1} is the gas constant, and

T = Thermodynamic (absolute) temperature

This equation gives the pressure on one side of the membrane.

Total pressure on the membrane is given by the difference between the pressures on the two sides. In animal cells, which lack a cell wall, excessive osmotic pressure can result in **cytolysis**.

Reverse Osmosis

Reverse osmosis (filtering), a process commonly used to purify water, is based on the principle of osmotic pressure:

- Water to be purified, is placed in a chamber and put under an amount of pressure, greater than the osmotic pressure exerted by the water and the solutes dissolved in it.
- Part of the chamber opens to a differentially permeable membrane that lets water molecules through, but not the solute particles.

DIALYSIS

Dialysis is the process of separating molecules in solution by the differences in their rates of diffusion through a semipermeable membrane, such as a dialysis tubing.

General Principle

- A solution containing several types of molecules, e.g. a **colloidal solution** which is made up of small sized particles that may not settle down under force of gravity and have much slower rates of diffusion (such as impurities of electrolytes and other soluble substances) **is placed into a semipermeable dialysis bag**, such as a cellulose membrane with pores, and the bag is sealed.
- The sealed dialysis bag is **placed in a container** of a different solution or pure water.
- **Molecules that are small enough** to pass through the tubing (often water, salts and other small molecules) **tend to move into or out of the dialysis bag**, in the direction of decreasing concentration.
- **Larger molecules** (often proteins, DNA or polysaccharides) that have dimensions significantly greater than the pore diameter **are retained inside** the dialysis bag.

Use in Medicine

In medicine, **dialysis** is a process for removing waste and excess water from the blood and is used, primarily, as an artificial replacement for the lost kidney function in people with renal failure.

- Dialysis is done by using a special fluid called **dialysate**.
- Dialysate is a mixture of pure water and chemicals.
- It is carefully controlled to pull wastes out of the blood without removing substances the body needs.
- A semipermeable membrane keeps the blood apart from the dialysate.
- This membrane lets the wastes and fluid in the blood flow through into the dialysate.

Types of Kidney Dialysis

There are two main types of kidney dialysis. These are referred to as hemodialysis and peritoneal dialysis.

Hemodialysis

In hemodialysis:

- Blood from a peripheral artery of the patient is allowed to flow on one side of a semipermeable membrane while an artificially prepared fluid of known solute concentration, called **dialysate**, flows by the other side but in opposite direction.
- Proteins present in the blood are the large nondiffusible ions.
- The solute concentration in the dialysate is so designed as to maximize diffusion of water and solutes across the membrane.
- The net effect is the reduction of toxic molecules to low levels in the blood while improving the levels of the essential ones.
- The purified blood is returned through a peripheral vein (Fig. 11.2).

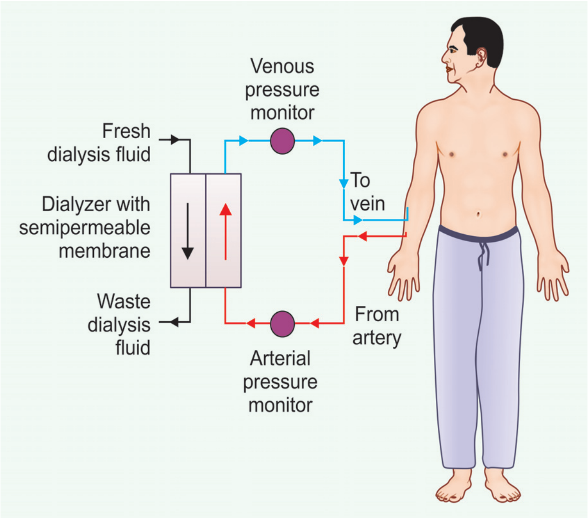


Fig. 11.2: Hemodialysis

Peritoneal Dialysis

In peritoneal dialysis the basic procedure is the same. In this case:

- The dialysate is run through a tube into the peritoneal cavity and the peritoneal membrane itself serves as the semipermeable membrane.
- The dialysate is retained for some time and eventually drained out through the tube, removing the excess of water and the waste products.

SURFACE TENSION

Surface tension is defined as the **ratio of the surface force (*F*) to the length (*d*) along which the force acts**. It is the elastic tendency of liquids which makes them acquire the least surface area possible.

- It is a **phenomenon in which the surface of a liquid**, where the liquid is in contact with gas, **acts like a thin elastic sheet**.
- This term is typically used only when the liquid surface is in contact with gas (such as the air).
- At liquid air interface, surface tension results from the greater attraction of water molecules to each other (due to cohesion) than to the molecules in the air (due to adhesion).
- The net effect is an inward force at its surface that causes water to behave as if its surface were covered with a stretched elastic membrane. These forces bind the surface particles together, though this binding is weak and is easy to break the surface of a liquid.
- Because of the relatively high attraction of water molecules for each other, water has a high surface tension (72.8 millinewtons per metre at 20°C) compared to that of most other liquids.
- Surface tension is an important factor in the phenomenon of capillary.

Units of Surface Tension

Surface tension is measured in SI units of N/m (newton per metre).

Causes of Surface Tension

Various intermolecular forces, such as van der Waals forces, draw the liquid particles together.

- Along the surface, the particles are pulled toward the rest of the liquid. Thus, the cohesive forces among liquid molecules are responsible for the phenomenon of surface tension.
- In the bulk of the liquid, each molecule is pulled equally in every direction by neighboring liquid molecules, resulting in a net force of zero. The molecules at the surface do not have the same molecules on all sides of them and therefore are pulled inward. This creates some internal pressure and forces liquid surfaces to contract to the minimal area.

Effects of Surface Tension

Surface tension is responsible for the shape of liquid droplets. Several effects of surface tension can be seen with ordinary water:

- When using a water dropper, the water does not flow in a continuous stream, but rather in a series of drops. Although easily deformed, droplets of water tend to be pulled into a spherical shape by the imbalance in cohesive forces of the surface layer.
- In the absence of other forces, including gravity, drops of virtually all liquids would be approximately spherical. The reason why the drop of water is not completely spherical is because of the force of gravity pulling down, on it.
- In the absence of gravity, the drop would minimize the surface area in order to minimize tension, which would result in a perfectly spherical shape.
- The spherical shape minimizes the necessary wall tension of the surface layer.
- The shape of the drop is caused by the surface tension of the water.
- Separation of oil and water is caused by a tension in the surface between dissimilar liquids. This type of surface tension is called interface tension.

Surfactants

Surface tension is visible in several phenomena, especially when surfactants are used to decrease it:

- Soap bubbles have very large surface area with very little mass. Bubbles in pure water are unstable. The addition of surfactants, however, can have a stabilizing effect on the bubbles. This is due to the reason that surfactants actually reduce the surface tension of water.
- Emulsions are a type of solution in which surface tension plays a role. Tiny fragments of oil suspended in pure water will spontaneously assemble themselves into much larger masses. But the presence of a surfactant provides a decrease in surface tension, which permits stability of minute droplets of oil in the bulk of water (or vice versa).

COLLOIDS

A colloid is a type of mixture in which a homogeneous noncrystalline substance, consisting of large molecules, is suspended through other substance in which the dispersed particle measures between 1 and 1000 nanometer. Particle of this size is not visible to naked eye and is distributed uniformly in solution. The particle (distributed) is referred as being in **dispersed phase** while the substance in which it is dispersed is called **continuous phase**.

If the particle size is smaller than 1 nm, the substance is known as **solution**. On the other hand, if the particle size is above 1000 nm, the substance is referred to as **suspension**.

Different types of colloids can be made, depending on the state of dispersed phase and continuous phase. These include sol, emulsion, foam and aerosol. **Sol** is formed with solid particles in liquid continuous phase. In **emulsion**, both phases are liquid. **Foam** is formed by gas dispersed in liquid or gas. On the other hand, **aerosol** contains liquid or solid, dispersed in gas.

Properties of Colloids

Colloids show:

- **Tyndall effect:** When a beam of light is allowed to pass through a colloidal solution, the path of light gets illuminated.
- **Brownian movement:** Zigzag movement of colloidal particles due to continuous collisions amongst themselves and also the molecules of dispersion medium.
- **Colligative property:** It depends on the number of moles of solute present in a given mass of solvent and colloidal particles being aggregates give low value of osmotic pressure, depression in freezing point and elevation in boiling point.
- **Electrophoresis:** Migration of colloidal particles to oppositely charged electrode under the influence of electric field.
- **Electro-osmosis:** When electrophoresis of dispersed particle in a colloidal system is prevented by some means, the dispersion medium itself begins to move in an electric field.

DONNAN MEMBRANE EQUILIBRIUM

Donnan membrane equilibrium, also called **Gibbs-Donnan membrane equilibrium** or **Gibbs-Donnan effect**, refers to the behavior of charged particles in a semipermeable membrane that sometimes fail to distribute evenly across two sides of the membrane.

- **It works on the principle** that when two solutions of differing concentrations are separated by a **semipermeable membrane**, their concentrations equalize as a result of **diffusion**.
- Diffusion occurs when substances move from area of high concentration to low concentration, down a concentration gradient.
- However, if there is an impermeable solute in one of the solutions, such as albumin, concentration of the solution does not equalize and that the concentration of the solution with impermeable solute remains high even at equilibrium.
  - Suppose a membrane is impermeable to anion R of a salt NaR in solution on one side of the membrane, but permeable to all other ions and salts involved.
  - In the beginning of the experiment, NaR is on one side of the membrane and NaCl on the other, as shown:

Na^+	Na^+
R^-	Cl^-
(1)	(2)

  - NaCl will diffuse from compartment (2) to (1) until equilibrium conditions are reached, so that:

Na^+	Na^+
R^-	
Cl^-	Cl^-
(1)	(2)

  - At equilibrium the product of the concentrations of a pair of diffusible ions (Na^+ and Cl^-) on one side of the membrane will be the same as the product of the concentration of the same ions on the other side of the membrane, so that:

    $(Na^+)1 \times (Cl^-)1 = (Na^+)2 \times (Cl^-)2$

  - The concentration of Na^+ in (1) must be equal to the sum of anions present ($R^- + Cl^-$), whereas on the other side the concentration of Na^+ is the same as that of the anion (Cl^-). This means that the concentration of Na^+ ions on the left must be greater than on the right and reverse is true for the Cl^- ions, i.e.

    $(Na^+)1 > (Na^+)2$ and $(Cl^-)1 < (Cl^-)2$

  - Hence, it is possible to account for the existence of different concentrations of diffusible ions on the two sides of a semipermeable membrane, the essential condition being the existence of nondiffusible ions on one side of a membrane, which is permeable to the other ions (Fig. 11.3).

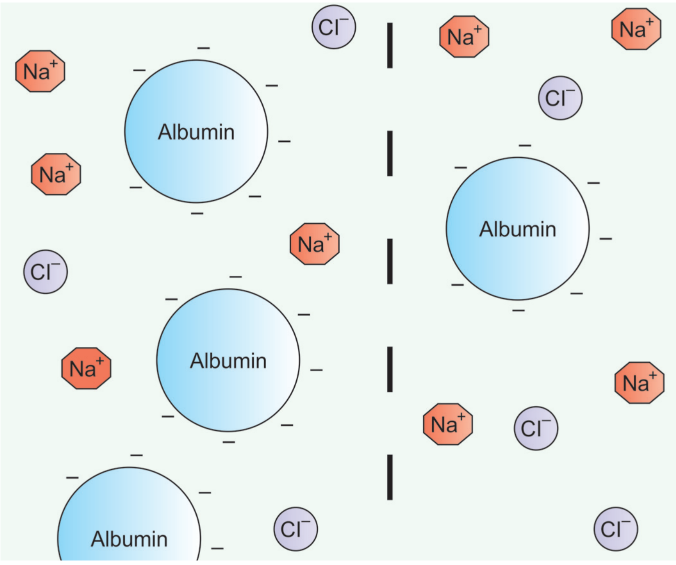


Fig. 11.3: Donnan membrane equilibrium

Clinical Usefulness of the Donnan Membrane Equilibrium

The principle of Donnan equilibrium has great importance in physiology while explaining diffusion of ions in a colloidal system through a membrane. The phenomenon is based upon ion activity existing in solutions.

- All living cells, as we know, contain impermeable anionic colloids, which are mostly made up of proteins and organic phosphates that cannot cross the cell membrane.
- As a result of it, there is a high concentration of nondiffusible anions across the cell membrane, thus, creating the Donnan equilibrium. This means that there are more ions inside the cell than outside.
- When a semipermeable membrane separates a nondiffusible substance such as protein, from diffusible substances, the diffusible anions and cations are distributed on the two sides of the membrane, so that the products of their concentrations are equal and that the sum of the diffusible and nondiffusible anions on either side of the membrane is equal to the sum of the concentrations of diffusible and nondiffusible cations.
- The unequal distribution of diffusible ions, thus produced, creates a potential difference across the membrane (membrane potential).

Donnan equilibrium accounts for the existence of gastric juice and other secretions, certain aspects of absorption and elimination, and difference in concentration of diffusible ions between plasma and red cells.

Important Question

1. Write notes on:
   i. Osmosis
   ii. Dialysis
   iii. Surface tension
   iv. Colloids
   v. Donnan membrane equilibrium
   vi. Osmotic pressure

Chapter 12

Reference Range

Reference range (**normal range**) for **blood tests** is a set of values used by a health professional to interpret a set of medical test results from blood samples. Reference range for blood tests is studied within the field of clinical chemistry (also known as clinical biochemistry, chemical pathology or pure blood chemistry), the area of pathology that is generally concerned with analysis of body fluids.

Blood test results should always be interpreted using the reference range provided by the laboratory that performed the test.

A **reference range** is usually defined as the set of values, 95% of the normal population falls within (that is, 95% prediction interval). It is determined by collecting data from vast numbers of laboratory tests.

In health-related fields, a reference range or reference interval is the range of values for a physiological measurement in healthy persons (for example, the amount of creatinine in the blood, or the partial pressure of oxygen). It is a basis for comparison (a frame of reference) for a physician or other health professional to interpret a set of test results for a particular patient.

The standard definition of a reference range (usually referred to, if not otherwise specified) originates in what is most prevalent in a reference group taken from the population. However, there are also optimal healthy range that appear to have the optimal health impact on people.

Values within the reference range are those within the normal distribution and are thus often described as within normal limits. The limits of the normal distribution are called the upper reference limit or upper limit of normal and the lower reference limit or lower limit of normal.

In healthcare-related publishing, style sheets sometimes prefer the word reference over the word normal to prevent the nontechnical senses of normal from being conflated with the statistical sense. Values outside a reference range are not necessarily pathological, and they are not necessarily abnormal in any sense other than statistically. Nonetheless, they are indicators of probable pathosis. Sometimes the underlying cause is obvious; in other cases, challenging differential diagnosis is required to determine what is wrong and thus how to treat it.

The standard definition of a reference range for a particular measurement is defined as the prediction interval between which 95% of values of a reference group fall into, in such a way that 2.5% of the time a sample value will be less than the lower limit of this interval, and 2.5% of the time it will be larger than the upper limit of this interval, whatever the distribution of these values.

Regarding the target population, if not otherwise specified, a standard reference range generally denotes the one in healthy individuals, or without any known condition that directly affects the ranges being established. These are likewise established using reference groups from the healthy population and are sometimes termed **normal range** or **normal values**. However, using the term normal may not be appropriate as not everyone outside the interval is abnormal and people who have a particular condition may still fall within this interval.

However, reference range may also be established by taking samples from the whole population, with or without disease conditions. In some cases, diseased individuals are taken as the population, establishing reference range among those having a disease condition. Preferably, there should be specific reference range for each subgroup of the population that has any factor which affects the measurement, e.g. specific range for each **sex**, **age group**, **race** or any other general determinant.

Normal Range Versus Reference Range

Normal range: As mentioned above, this term was earlier used to quote normal ranges for the concentration of the constituents in body fluids to indicate the values found in persons who were in good health and by inference to define results which indicate that some abnormality is present. However, using the term normal may not be appropriate as not everyone outside the interval is abnormal and that the people who have a particular condition may still fall within this interval. Now, it has been appropriately replaced with the term, reference range, which is sometimes still termed as normal ranges or normal values.

Reference range: Reference range, as discussed above, refers to a range of values of a particular quantity obtained from individuals in defined states of health. The reference group is taken from the general population. It is the interval between which 95% of values of a reference population fall into, in such a way that 2.5% of the time a value will be less than the lower limit of this interval and 2.5% of the time it will be larger than the upper limit of this interval, whatever the distribution of these values may be? Various physiological factors are taken into account while establishing and interpreting the reference range, like age, gender, pregnancy, time of sample withdrawn for some of the parameters, etc.

Reference Range of Some Constituents in Blood

Reference range of some constituents in blood is given in Table 12.1.

Reference Range of Acid–Base Parameters

If arterial/venous is not specified for an acid–base or blood gas value, then it generally refers to arterial, and not venous, which otherwise is standard for other blood tests. Acid–base and blood gases are among the few blood constituents that exhibit substantial difference between arterial and venous values. Reference range of acid–base parameters is given in Table 12.2.

Reference Range of Hormones

Reference range of some hormones is given in Table 12.3.

Reference Range of Some Constituents in Urine

Reference range of some constituents in urine is given in Table 12.4.

Table 12.1 Reference range of some constituents of blood

Test	Lower limit	Upper limit	Unit
Sodium (Na)	135	145	mmol/L (mEq/L)
Potassium (K)	3.5	5.0	mmol/L (mEq/L)
Chloride (Cl)	95	105	mmol/L (mEq/L)
Total calcium (Ca)	8.5	10.5	mg/dL
Ionized calcium (iCa)	4.4	5.2	mg/dL
Ceruloplasmin	18	35	mg/dL
Inorganic phosphorus	2.5	4.5	mg/dL
Uric acid (males)	4.5	7.0	mg/dL
Urea	15	45	mg/dL
Creatinine	0.8	1.5	mg/dL
Total proteins	6.3	8.4	g/dL
Albumin	3.5	5.5	g/dL
Globulins	2.3	3.5	g/dL
Bilirubin, total	0.2	0.8	mg/dL
Bilirubin, conjugated	0.0	0.3	mg/dL
Alanine transaminase (ALT)/glutamate pyruvate transaminase (SGPT)	8	56	IU/L
Aspartate transaminase (AST)/glutamate oxaloacetate transaminase (SGOT)	8	40	IU/L
Alkaline phosphatase	39	117	U/L
Gamma-glutamyl-transferase (GGT)	5	35	U/L
Creatine kinase (CK)	20	50	IU/L
Triglycerides (below 40 years of age)	54	110	mg/dL
Triglycerides (above 40 years of age)	70	150	mg/dL
Total cholesterol	120	200	mg/dL
HDL cholesterol, male	35	80	mg/dL
HDL cholesterol, female	40	86	mg/dL
LDL cholesterol	80	120	mg/dL
Glucose	60	90	mg/dL
Glycated hemoglobin (HbA1C)	4.0	6.0	% of Hb
Lactate dehydrogenase (LDH)	50	150	U/L
Amylase	53	190	U/L
Acid phosphatase	—	3	U/L

Table 12.2 Reference range of acid–base parameters

Test	Lower limit	Upper limit	Unit
pH	7.35	7.45	
$[H^+]$	36	44	nmol/L
Base excess	–3	+3	mEq/L
pO_2	75	100	mm Hg
Oxygen saturation	95	100	%
pCO_2	35	45	mm Hg
HCO_3^-	18	23	mmol/L

Table 12.3 Reference range of some hormones

Test	Lower limit	Upper limit	Unit
Thyroid stimulating hormone (TSH)	0.3	5.0	mIU/L
Free thyroxine (FT_4)	0.89	1.76	ng/dL
Total thyroxine (T_4)	4.5	12.5	mg/dL
Free tri-iodothyronine (FT_3)	2.3	4.2	pg/mL
Total tri-iodothyronine (T_3)	86	187	ng/dL

Table 12.4 Reference range of some constituents in urine

Constituent	Normal excretion
D-xylose	1–2 g/5 hours
Total nitrogen	10–20 g/24 hours
Creatinine	1.0–2.0 g/24 hours
Calcium	0.1–0.7 g/24 hours
Chloride	110–250 mEq/24 hours
Sodium	40–220 mEq/24 hours
Potassium	25–125 mEq/24 hours
Phosphorus	0.4–1.3 g/24 hours
Urea	25–30 g/24 hours
Uric acid	0.5–1.0 g/24 hours

Reference Range of Some Constituents in CSF

Reference range of some constituents in CSF is given in Table 12.5.

Table 12.5 Reference range of some constituents in CSF

Constituent	Normal concentration
Total protein	20–40 mg/dL
Glucose	50–75 mg/dL
Chloride (as NaCl)	700–750 mg/dL (118–132 mmol/L)

Reference Range of Some Parameters of Gastric Juice

Reference range of some parameters of gastric juice is shown in Table 12.6.

Table 12.6 Reference range of some parameters of gastric juice

Parameter	Normal range
Volume	2–3 L/24 hours
pH	1.6–1.8
Titrable acidity (fasting sample)	15–35 mEq/L
Basal acid output	
Males	1.0–5.0 mEq/hr
Females	0.2–3.8 mEq/hr
Maximal acid output (after stimulation with histamine/pentagastrin)	
Males	18–28 mEq/hr
Females	11–21 mEq/hr

Important Questions

1. Define reference range.
2. Give reference range of:
   i. Blood glucose
   ii. Serum cholesterol
   iii. Serum calcium
   iv. Serum uric acid
   v. T3, T4 and TSH

Chapter

13

Laboratory Glass Apparatus

Glasswares and glass apparatus refer to a variety of equipment, traditionally made of glass, used for scientific experiments. They have **wide variety of functions**.

Types of Glass used for Making Glass Apparatus

Type of glass used for manufacturing various glass apparatuses depends upon the application. Commonly used glass is given below.

Borosilicate Glass

It is a type of glass with silica and boron trioxide as the main glass forming constituents. Borosilicate glasses are known for having very low coefficients of thermal expansion, making them resistant to thermal shock than any other common glass. Since it is subjected to thermal stress, borosilicate glass is commonly used for the construction of laboratory glassware. It is generally sold under the trade name Borosil.

Fused Quartz Glass

Fused quartz or fused silica is the type of glass consisting of silica in amorphous (noncrystalline) form. It differs from traditional glass in the way that it has no other ingredients which are typically added to the glass to lower the melting temperature. Thus, fused silica has high working and melting temperatures. Accordingly, optical and thermal properties of the fused quartz are superior to the other types of glass due to its purity. Quartz glass is commonly used in cuvettes since it is transparent in certain parts of the electromagnetic spectrum.

Darkened-brown Glass

Darkened-brown or amber glass is commonly used in storage bottles. It can block ultraviolet and infrared radiations.

Types and Applications of Laboratory Glass Apparatus

Glass apparatuses have a **wide variety of functions**, which include volumetric measurements, holding or storage of chemicals and samples, mixing or preparing solutions, carrying out of the various laboratory processes like chemical reactions, heating and cooling, etc. distillation, separations including chromatography, growing biological organisms, etc.

TEST TUBES

A test tube, also known as a **culture tube** or **sample tube**, is a common piece of laboratory glassware consisting of a finger-like length of glass or clear plastic tubing, open at the top, usually with a rounded U-shaped bottom.

- These are **cylindrical** pieces of glassware used to hold, mix, heat or transport small quantities of solid or liquid chemicals, especially for qualitative experiments and assays.
- A large test tube specifically for boiling liquids is called a **boiling tube**.
- Test tubes are available in a **multitude of lengths and widths**, typically from 10 to 20 mm wide and 50 to 200 mm long.
- The top often features a **flared lip** to aid pouring out the contents.
- Some sources consider that the presence of a lip is what distinguishes a test tube from a **culture tube**.
- A test tube has either a flat bottom, a round bottom or a conical bottom.
- Some test tubes are made to accept a ground glass stopper or a screw cap. They are often provided with a small ground glass or white glaze area near the top for labelling with a pencil.

Types of Tubes

Based on the need of an experiment, different types of tubes are used in a clinical chemistry laboratory. These are discussed below.

With or Without Rim

These are of uniform thickness and withstand mechanical and thermal shocks. Tubes with rim have a small rim at the top and are preferred when reagent in tube is directly heated on the flame. The rim helps in holding the tube with a test tube holder (Fig. 13.1).

Commonly used test tubes are of the following specifications:

Outer diameter (mm) × Length (mm)

1. 10 × 75: These are used for testing procedures as well as for centrifugation.
2. 15 × 125: These are used for most of the biochemistry tests.
3. 18 × 150: These are used for heating the reaction mixture directly on flame.

Centrifuge Tubes

These are either graduated or plain and are conical in shape at the bottom. They may be stoppered or nonstoppered (Fig. 13.2). Commonly used tubes are of the size of 17 × 120 mm.

Folin-Wu Tubes

These tubes are mainly used for the determination of blood sugar by Folin-Wu method. These are engraved at 12.5 and 25 mL (Fig. 13.3).

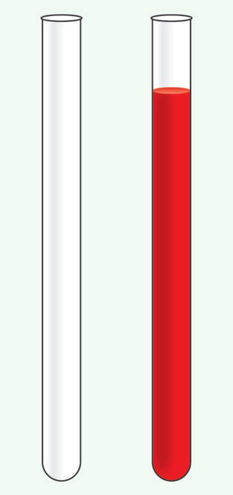


Fig. 13.1: Test tubes with and without rim

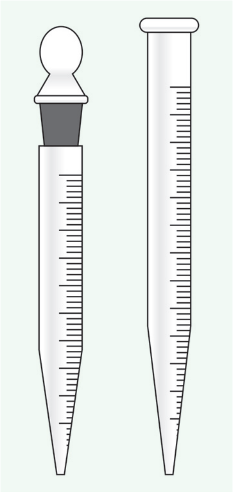


Fig. 13.2: Centrifuge tubes—stoppered and nonstoppered

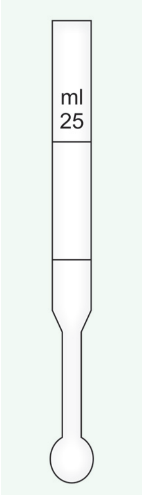


Fig. 13.3: Folin-Wu tube

BEAKERS

Beakers are simple containers used mainly for the preparation of solutions. These are generally cylindrical in shape, with a flat bottom. Most also have a small **spout** or **beak** to aid pouring (Fig. 13.4).

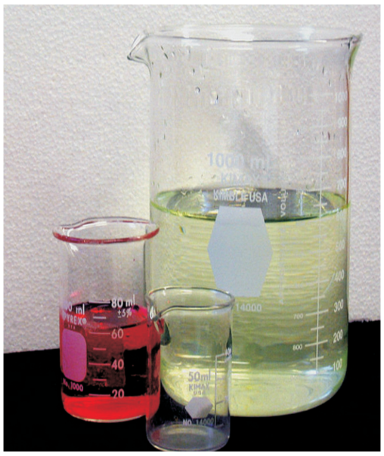


Fig. 13.4: Beakers

- Beakers are available in a wide range of capacities from 5 to 5000 mL.
- They are often **graduated**, i.e. marked on the side, with lines indicating the volume contained. For instance, a 250 mL beaker might be marked with lines to indicate 50, 100, 150, 200 and 250 mL of volume. These marks are not intended for obtaining a precise measurement of volume, like a graduated cylinder or a volumetric flask, which would be more appropriate for such a task, but rather an estimation.
- Most beakers are accurate to within ~10%.

FLASKS

Flasks are narrow-necked glass containers, typically conical or spherical in shape. These are used in a laboratory to hold reagents or samples.

- Flasks come in a number of shapes and a wide range of sizes but a common distinguishing aspect in their shapes is a **wider vessel body** and one, or sometimes more, **narrow tubular** sections at the top called **neck**, which have an opening at the top. Their sizes are specified by the volume they can hold and may vary from 25 to 5000 mL.
- Flasks can be used for making solutions or for holding, containing, collecting or sometimes volumetrically measuring chemicals, samples, solutions, etc. for chemical reactions or other processes such as mixing, heating, cooling, dissolving, precipitation, boiling (as in distillation), or analysis.

- At the opening, at top of the neck of some glass flasks, such as round-bottom flasks and volumetric flasks, there are outer (or female) tapered (conical) ground glass joints.
- Some flasks, especially volumetric flasks, come with a **stopper** or cap for capping the opening at the top of the neck. Glass stoppers typically have a matching tapered inner (or male) ground glass joint surface but often only of stopper quality. Flasks which do not come with such stoppers or caps included may be capped with a rubber or cork stopper.

Types of Laboratory Flasks

There are several types of laboratory flasks, all of which have different functions within the laboratory. Flasks, because of their use, can be divided as shown below.

Conical Flasks (Erlenmeyer Type)

These are shaped like a cone, usually completed by the ground joint. These are used for performing titrations and for boiling the solutions. Because of the conical shape, evaporation is minimum. These are very popular because of their low price (since they are easy to manufacture) and portability (Fig. 13.5).

Volumetric Flask

It is used for preparing liquids with volumes of high precision. They are flat bottomed, pear-shaped with long narrow neck that has a specific volume mark and fitted with a stopper. These are mainly used to make final volume of the reagent very accurately (Fig. 13.6).

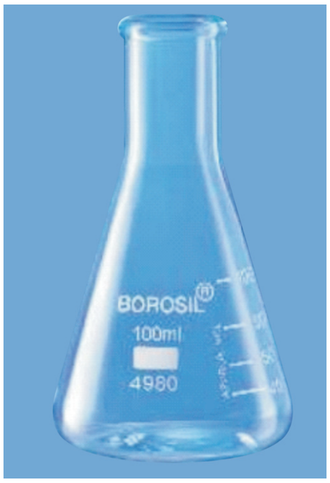


Fig. 13.5: Erlenmeyer flask

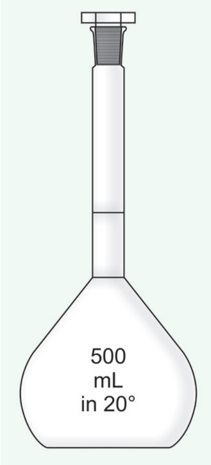


Fig. 13.6: Volumetric flask

Round Bottom Flasks

These are usually spherical, i.e. round-bottom, and have a long neck (Fig. 13.7).

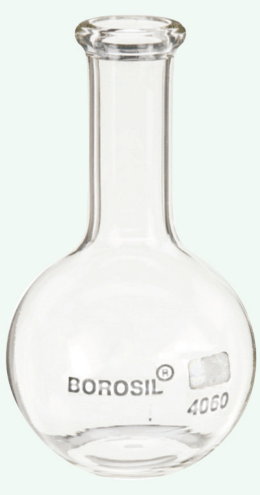


Fig. 13.7: Round bottom flask

Distillation Flasks

These are intended to contain mixtures, which are subject to distillation as well as to receive the products of distillation. Distillation flasks are available in various shapes. Similar to the reaction flask, distillation flasks have a narrow neck with the ground glass joint. However, these are made of thinner glass than the reaction flask, so that it is easier to heat. They are sometimes spherical, test tube-shaped or pear-shaped, also known as a Kjeldahl flask, due to its use with Kjeldahl bulbs.

They may have one, two or more necks, each topped by ground glass connections. They are used in more complex reactions that require the controlled mixing of multiple reagents (Fig. 13.8).

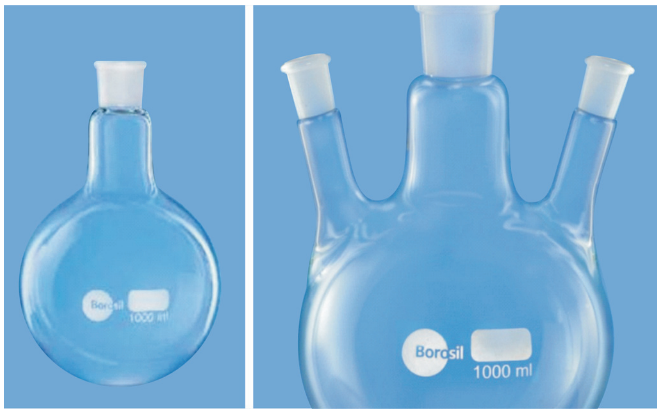


Fig. 13.8: Distillation flasks

Buchner Flask or Sidearm Flask

It is a thick-walled conical flask with a short hose-connection tube on the side of the neck (Fig. 13.9).

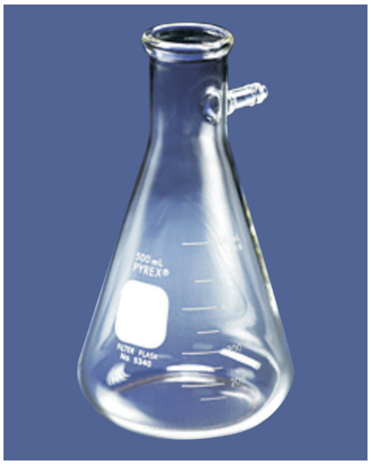


Fig. 13.9: Buchner flask

MEASURING CYLINDERS

A measuring cylinder, **graduated cylinder** or **mixing cylinder** is a piece of laboratory glassware, which is generally used to measure the volume of a liquid. Graduated cylinders are generally more accurate and precise than laboratory flasks or beakers. However, they are less accurate and precise than the volumetric flasks or pipettes (Fig. 13.10).

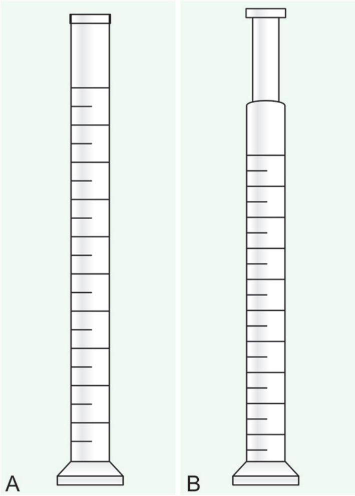


Fig. 13.10: Measuring cylinders: (A) Unstoppered; (B) Stoppered

- A traditional graduated cylinder is usually **narrow** and **long**, and has a glass **bottom** and a **spout** for easy pouring from the measured liquid.
- Some of the cylinders have **ground glass joint** instead of a spout so that they can be closed with a **stopper**. They are also known as **mixing cylinders**.
- A **graduated cylinder** is meant to be read with the surface of the liquid at eye level where the center of the meniscus shows the measurement line. Typical capacities of graduated cylinders are between 5 mL and 2 liters.
- Graduated cylinders are sometimes used to indirectly measure the volume of a solid by measuring the displacement of a liquid.

PIPETTES

A pipette, pipet, pipettor or chemical dropper, is a laboratory tool, commonly used in a chemistry, biology or medicine, to transport a measured volume of liquid, often as a media dispenser. Pipettes come in several designs for various purposes with differing levels of accuracy and precision, from single piece glass pipettes to more complex adjustable or electronic pipettes which work by creating a partial vacuum above the liquid-holding chamber and selectively releasing this vacuum to draw up and dispense the desired quantity of liquid. Measurement accuracy varies depending on the style.

Types of Pipettes

These are classified as follows.

Graduated Pipettes

Graduated pipettes are a type of pipette consisting of a long tube with a series of graduations, as on a graduated cylinder or burette, to indicate different calibrated volumes.

- They require a source of vacuum; for which purpose, the mouth is used. However, to avoid accidental ingestion of potentially harmful substances, these days, a variety of **pro-pipetters** have been developed, both entirely manual and electrically assisted.
- Graduated pipettes have +/– tolerances that range from 0.6% to 0.4% of the nominal volume, when measured at 20°C. These are manufactured according to ISO specifications for accuracy and the arrangement of the graduations. These are available from 0.1 to 10 mL capacities. The graduations are durable, resistant to chemical attack and normal washing.
- They are available, both in Class A and B accuracies. Class A pipettes are very accurate and used for quantitative determinations.

Graduated pipettes are of two types:

- **Mohr pipettes:** A Mohr pipette, also called **backward** or **drain-out pipette**, is a type of pipette used to measure the volume of the liquid dispensed, although not as accurately as a volumetric pipette. It was invented by Karl Friedrich Mohr, the Father of Volumetric Analysis. These have a series of marked lines (as on a graduated cylinder) to indicate the different volumes. They come in a variety of sizes and are used much like a burette, in that the volume is found by calculating the difference of the liquid level before and after (Fig. 13.11).

These are **graduated above the tip**. These can be used even if the tip is broken. These are, however, not convenient to perform serological tests. In this type of pipette, a volume is pulled into the pipette, sometimes to the maximum volume, then the needed volume is aliquoted out.

- **Serological pipettes:** These are **graduated up to the tip**. These are mainly used to perform serological tests and for pipetting specimen like blood, serum, plasma as well as standard solutions (Fig. 13.12).

Volumetric Pipettes

Volumetric pipettes or **bulb pipettes** are not graduated but designed specifically to deliver a specific quantity of the specimen. These pipettes have a large bulb with a long narrow portion above, with a single graduation mark, as it is calibrated for a single volume (like a volumetric flask). Typical volumes are 10, 25 and 50 mL. Volumetric pipettes are commonly used to make laboratory solutions from a base stock as well as prepare solutions for titration (Fig. 13.13).

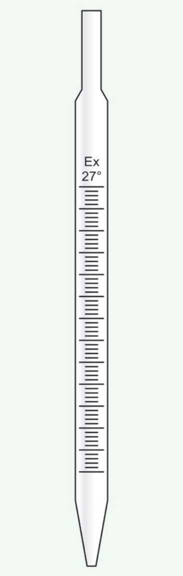


Fig. 13.11: Mohr type pipette

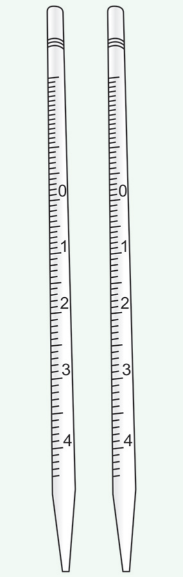


Fig. 13.12: Serological pipettes

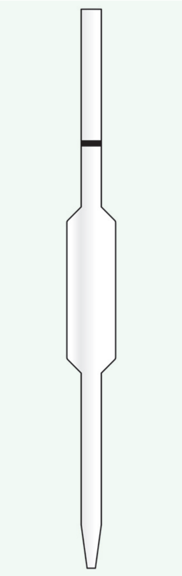


Fig. 13.13: Volumetric pipette

Pasteur Pipettes

These are plastic or glass pipettes used to transfer small amounts of liquids, but are not graduated or calibrated for any particular volume. The bulb is separate from the pipette body. Pasteur pipettes are also called **teat pipettes**, **droppers**, **eye droppers** or **chemical droppers**.

These pipettes can be prepared in the laboratory by using glass tubing. These are used by attaching rubber teats for the separation of serum and plasma from the cells. These are also useful in qualitative determinations where few drops of specimen are required to perform a test (Fig. 13.14).

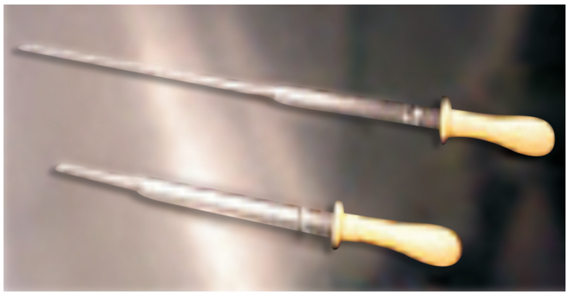


Fig. 13.14: Pasteur pipettes

BURETTES

A burette or **buret** is a long graduated tube of uniform bore which is closed at the lower end by means of a glass stopcock. They are used in analytical chemistry for titrations and to dispense variable volumes of the corrosive reagents. They are available in capacities of 1–100 mL (Fig. 13.15).

A burette is distinguished from a pipette by the fact that the quantity delivered is variable. Thus, in a titration, one solution is dispensed with a pipette and another solution is added to it from a burette in aliquots of varying size.

A traditional burette consists of glass tube of constant bore with a graduation scale on it and a stopcock at the bottom. The barrel of the stopcock may be made of glass or plastic. Stopcocks with glass barrels need to be lubricated with Vaseline or a specialized grease.

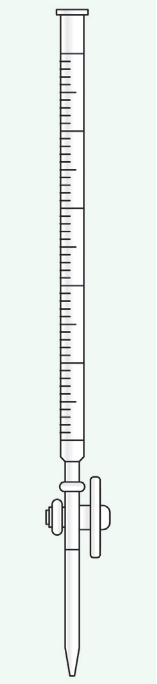


Fig. 13.15: Burette

Burettes are manufactured to specified tolerances, designated as class A or B, which is also marked on the glass.

Burettes may be designated for use at a particular temperature. If used at another temperature they should be subjected to calibration.

FUNNELS

These are used to get materials through a narrow opening. There are many different kinds of funnels that have been adapted for the specialized applications. These include filter funnels, thistle funnels (shaped like thistle flowers) and dropping funnels have stopcocks, which allow the fluid to be added to a flask slowly. When used with filter paper, **filter funnels** and **Buchner funnels** can be used to remove fine particles from a liquid in a process called filtration. For more demanding applications, the filter paper in the latter two may be replaced with a **sintered glass**. **Separatory funnels** are used in liquid/liquid extractions.

Types of Funnels

Plain funnels **exist in various dimensions with long or short necks. The commonly used funnels are of diameters of 50, 65, 75 and 100 mm**.

Filtration Funnels

Filter funnels have a neck of a thin capillary tube and rib which increase the filter papers' effectiveness and thus accelerate the process of filtration (Fig. 13.16).

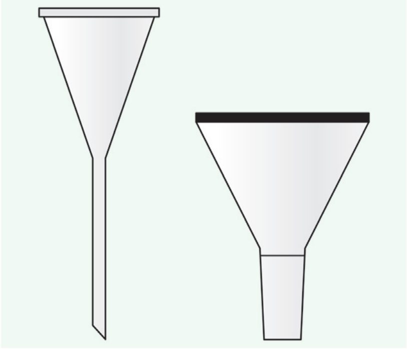


Fig. 13.16: Filtration funnels

Separatory Funnels

Separatory funnels are pear-shaped, have a cap and a short neck, with a stopcock for the even pouring of fluids. These are used to decant two immiscible fluids. The two layers formed are usually known as the organic and aqueous phases. Most organic solvents float on top of an aqueous phase (Fig. 13.17).

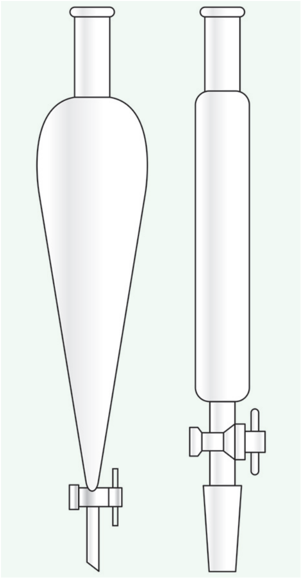


Fig. 13.17: Separatory funnels

Buchner Funnels

Buchner funnels are made of porcelain and include a plate of sintered glass or perforated porcelain. These are used in filtration under low pressure with a Buchner flask by vacuum suction (Fig. 13.18).

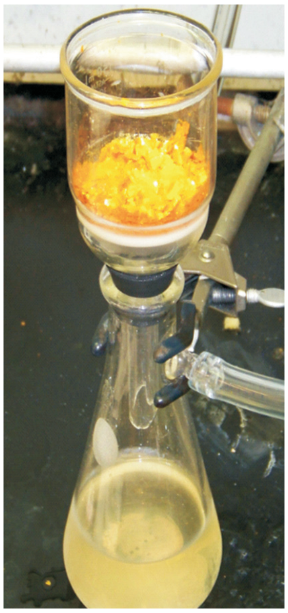


Fig. 13.18: Buchner funnel

BOTTLES

These are containers with narrow openings, generally used to store reagents or samples. Small bottles are called vials.

Types of Bottles

Different types of bottles used are as follows.

Reagent Bottles

They are available in 25–5000 mL capacities. They are cylindrical, have narrow necks and fitted with stoppers. These are made up of plain glass or amber-colored glass. Amber-colored bottles are useful for the storage of certain type of reagents, like silver nitrate, which are light sensitive (Fig. 13.19).

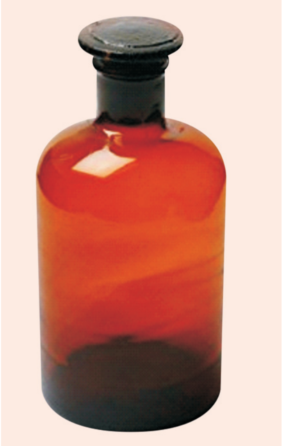


Fig. 13.19: Reagent bottle

Screw-cap Bottles

These are available in 5–1000 mL capacities and may be round or flat. Their caps may be made up of metal or plastic. These are good to store hygroscopic reagents (Fig. 13.20).

Drop Bottles

These are of about 50–100 mL capacities and made in white or brown glass with a narrow neck and a slotted glass stopper. They are used for delivery of drops of solutions, such as stains (Fig. 13.21).

STIRRING ROD

A stirring rod or **stir rod** is a piece of laboratory glassware, used to mix chemicals and liquids in a laboratory. They are usually made of solid glass, about the thickness and slightly longer than a drinking straw, with rounded ends (Fig. 13.22).

GLASS TUBING

Glass tubing is a hollow piece of borosilicate or flint glass used primarily as laboratory glassware. Glass tubing is commercially available in various thicknesses and lengths. For use in the laboratory, glass tubing is attached to rubber stoppers.

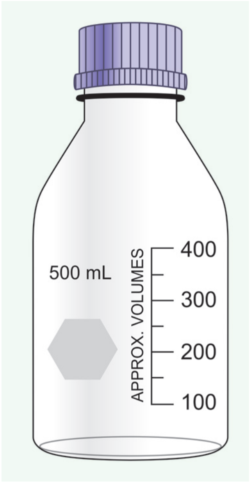


Fig. 13.20: Screwcap bottle

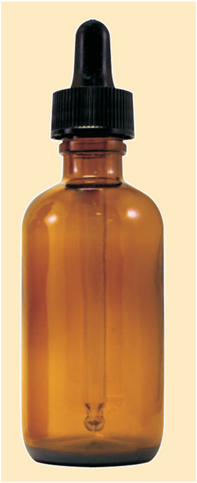


Fig. 13.21: Drop bottle

Fig. 13.22: Stirring rod

PETRI DISH

A Petri dish, alternatively known as a **Petri plate** or **cell-culture dish**, named after the German bacteriologist Julius Richard Petri, is a shallow cylindrical glass or plastic lidded dish that biologists use to culture cells, such as bacteria (Fig. 13.23).

Modern Petri dishes, usually, feature rings and/or slots on their lids and bases so that when stacked, they are less prone to sliding off one another. Multiple dishes can also be incorporated into one plastic container to create a **multi-well plate**.

While glass Petri dishes may be reused after sterilization, such as in an autoclave or one hour's dry heating in a hot air oven at 160°C, plastic Petri dishes are often disposed off after experiment where cultures might contaminate each other.

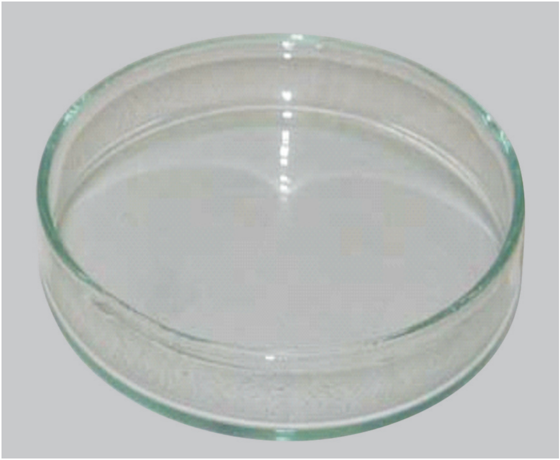


Fig. 13.23: Petri dish

SYRINGES

These are mainly used for the collection of blood and CSF. Most commonly used syringes are of 2, 5, 10 and 20 mL capacities.

Pipetting Syringes

Pipetting syringes are hand-held devices that combine the functions of volumetric (bulb) pipettes, graduated pipettes and burettes. They are calibrated to ISO volumetric A grade standards. A glass or plastic pipette tube is used with a thumb-operated piston and PTFE seal which slides within the pipette in a positive displacement operation. Such a device can be used on a wide variety of fluids (aqueous, viscous and volatile fluids; hydrocarbons; essential oils and mixtures) in volumes between 0.5 mL and 25 mL. This arrangement provides improvement in precision, handling safety, reliability, economy and versatility. No disposable tips or pipetting aids are needed with the pipetting syringe (Fig. 13.24).

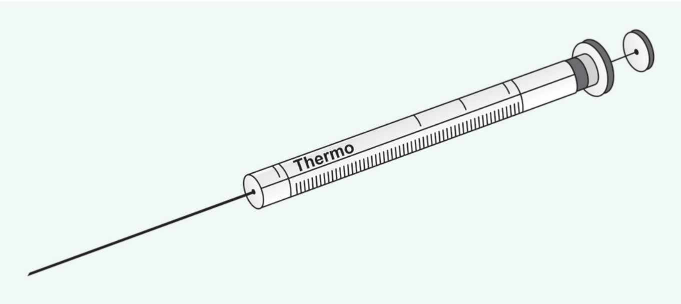


Fig. 13.24: Pipetting syringe

DISPENSERS

The term dispenser means repeated discharge of the defined quantity. For easy, rapid and precise dispensing of reagents, bottle top dispensers are widely employed. They can be mounted directly on the commercial laboratory bottles, either directly or by means of some adapter.

Thus, dispenser refers to a device for repeatedly delivering fixed volumes (from as small as 0.2 microliters up to 100 milliliters, depending on the model) of liquid, such as caustic or volatile solvents, like toluene, or growth medium, like agar, into a series of receptacles. They operate without biological or chemical contamination and are designed for easy cleaning, before and after use.

A dispenser has an integral syringe, which functions as a pipette and is mounted on top of a reagent bottle. They are intended for adding the reagent in a series of tubes.

The **syringe** is adjusted to any selected volume within the range of syringe calibration. It is filled from the **bottle** through a tube dipping into the reagent and is expelled through the delivery jet. The syringe plunger is depressed by hand to dispense the reagent and refilling of the syringe is usually achieved by a spring mechanism. A valve mechanism allows fluid to be aspirated from the bottle during filling, without air entering the dispenser tip and allows later expulsion through the tip without back flow into the bottle (Fig. 13.25).

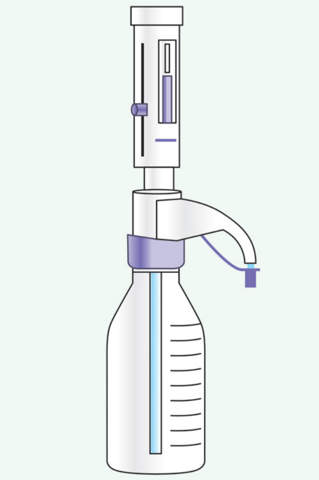


Fig. 13.25: Dispenser

Functional Principle of Bottle Top Dispensers

By an upward movement of the piston, a preset amount of the liquid is aspirated from the reagent bottle into the dispenser cylinder. By subsequent downward movement of the piston, liquid is released through a valve system and the discharge tube. There is no need to set a meniscus or to observe a waiting time.

The following precautions and safety must be taken while choosing and using a bottle top dispenser:

- Check suitability of the dispenser, for the liquid to be dispensed.
- Fully prime the syringe by drawing and dispensing sample to remove trapped air, which can cause inaccuracies.
- Slowly dispense the final sample volume into an appropriate receptacle.
- After use, rinse the syringe with an appropriate cleaning solvent, followed by water.
- It should be checked regularly, including re-calibration, greasing of the moving parts and replacement of any worn-out seals or other parts.

Mechanical dispensers operate in a manner similar to automatic dispensers except that the syringe plunger is driven mechanically by an electric motor. This offers more control over the ejection step and is less tiring for repetitive use.

MICROSCOPE SLIDES AND COVERSLIPS

A microscope slide is a thin flat piece of glass, typically 75 by 26 mm (3 by 1 inches) and about 1 mm thick, used to hold objects for examination under a microscope. Typically, the object is placed or secured (mounted) on the slide, and then both are inserted together in the microscope for viewing. This arrangement allows several slide-mounted objects to be quickly inserted and removed from the microscope, labeled, transported and stored in appropriate slide cases or folders.

Microscope slides are often used together with a **coverslip** or **cover glass**, a smaller and thinner sheet of glass that is placed over the specimen (Fig. 13.26).

Slides are held in place on the microscope's stage by slide clip, slide clamp, or a cross table, which is used to achieve precise, remote movement of the slide upon the microscope's stage (such as in an automated/computer operated system or where touching the slide with fingers is inappropriate either due to the risk of contamination or lack of precision).

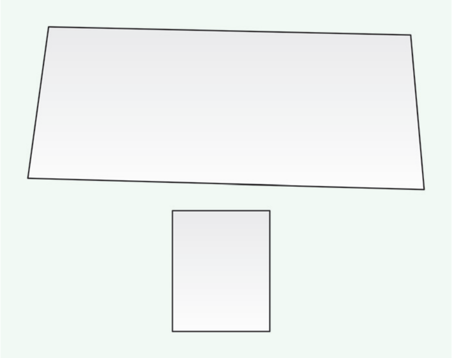


Fig. 13.26: Microscope slide and coverslip

JARS

These are cylindrical containers with wide openings that may be sealed.

CONDENSERS

A condenser is a piece of apparatus or equipment that can be used to condense, i.e. to change the physical state of a substance from its gaseous to the liquid state; in the laboratory. It is generally used in procedures done with organic liquids brought into gaseous state through heating or application of vacuum (lowered pressure), though processes often involve at least trace amounts of the common inorganic component water, and can involve other inorganic substances as well.

Condensers can be applied at various scales, from micro-scale (very few microliters) to process scale (many liters), using laboratory glassware and occasionally metalware that accomplish the cooling of the vapor generated by boiling (through heating or application of vacuum).

In the laboratory, condensers are generally used in procedures done with organic liquids brought into gaseous state through heating with or without lowering the pressure (applying vacuum).

Types of Condensers

These are available in various sizes and types.

Simple Condenser

In the simplest form, a condenser consists of a single tube of glass or metal, where the flow of outside air produces the cooling.

Liebig Type of Condenser

Liebig type of condenser consists of concentric glass tubes, with the tube through which the hot gases begin to pass, running the length of the apparatus. The second tube defines an outer chamber through which air, water or other cooling fluids can pass to reduce the temperature of the gases to afford the condensation; hence, the outer tube (or, as designs become more complex, outer cooling chamber) has an inlet and an outlet to allow the cooling fluid to enter and exit.

This type of apparatus is used for the preparation of glass-distilled water. A flat bottom flask is used with a side delivery tube and condenser. It can also be used for the redistillation of organic solvents like ethanol, isopropanol, etc.

Coil Condenser

A coil condenser has a spiral coil running the length of the condenser through which coolant flows and this coolant coil is jacketed by the vapor/condensate path.

Friedrich's Condenser

A Friedrich's condenser (Friedrich's condenser) is a spiral finger condenser, which consists of a large, spiral internal cold finger type capillary tube disposed within a wide cylindrical housing. Coolant flows through the internal cold finger; accordingly, vapors rising up through the housing must pass along the spiral path (Fig. 13.27).

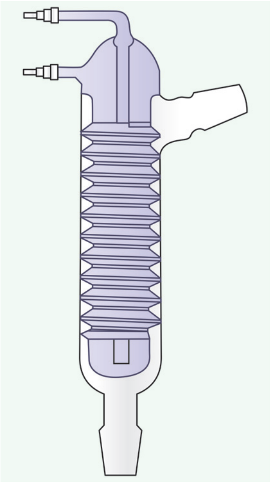


Fig. 13.27: Friedrich's condenser

DISTILLATION APPARATUS

These are used for purification of a low boiling liquid from higher boiling or nonvolatile impurities (Fig. 13.28).

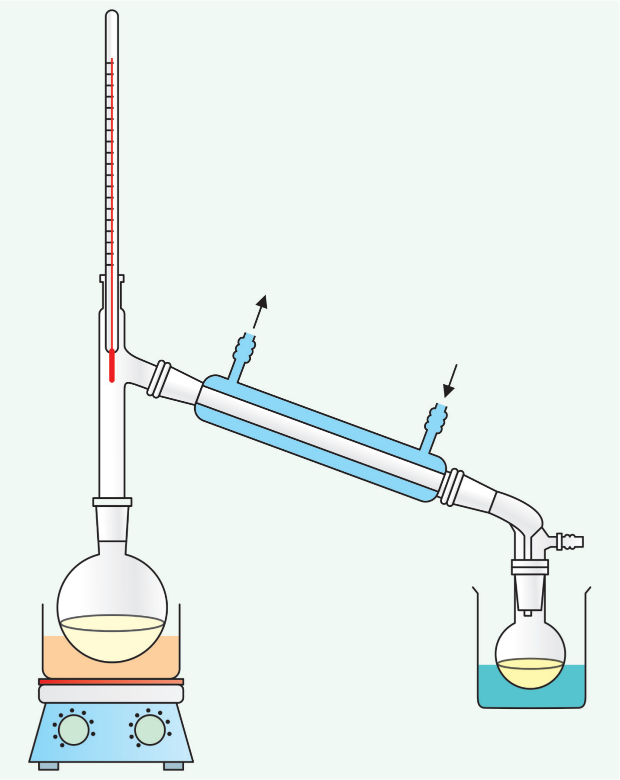


Fig. 13.28: Distillation apparatus

Impure liquid is placed in boiling flask and heated with stirring (or other assistance to boiling, to prevent bumping). Vapor rises diagonally to the right, coming in contact with a cold finger type of condenser, chilled by fluid flow through ports. As warm gaseous material, boiled from flask, comes in contact with cold surface of condenser, the gaseous material changes state to liquid (condenses and falls, guided by the offset, pointed tip of the cold finger, to the right, through a funnel tip into collection flask, which is correspondingly cooled, to prevent any loss of product through adventitious heating and evaporation.

LOW PRESSURE VACUUM DISTILLATION SYSTEMS

- Distillation under reduced pressure should always be carried out behind adequate shields.
- Always use a suitable trap between the sources of vacuum and the apparatus.
- Never subject the apparatus to rapid changes of pressure.
- At the end of distillation, allow it to cool, before slowly admitting air.

DESICCATORS

Desiccators are used to dry materials or keep them dry. These are sealable enclosures containing desiccants used for preserving moisture sensitive items. A common use for desiccators is to protect chemicals which are hygroscopic or which react with water from humidity, mainly the chemicals used for the preparation of accurate normal solution and standards (Fig. 13.29).

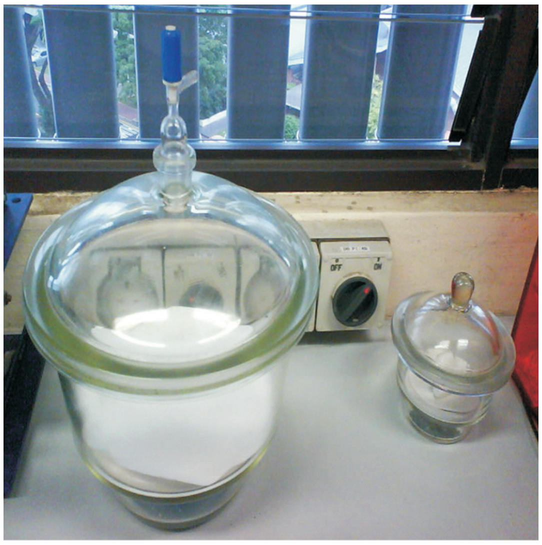


Fig. 13.29: Desiccators

The contents of desiccators are exposed to atmospheric moisture whenever the desiccators are opened. It also requires some time to achieve a low humidity. Hence, they are not appropriate for storing chemicals which react quickly or violently with atmospheric moisture, such as the alkali metals. Desiccators are sometimes also used to remove traces of water from an almost dry sample.

These are available in variable sizes. Chemicals like calcium chloride or silica gel, etc. are used as desiccants.

Vacuum Desiccators

Desiccators are sealable enclosures, containing desiccants, which are used for preserving moisture sensitive items. A common use for desiccators is to protect chemicals, which are hygroscopic or those which react with water from humidity. Contents of the desiccators are slowly exposed to atmospheric moisture, whenever the desiccators are opened.

In laboratory use, the most common desiccators are circular in shape and are made up of heavy glass. It has two parts, i.e. a storage chamber and a lid. Further, the storage chamber has two compartments:

The **lower compartment** of the desiccator contains lumps of silica gel, freshly calcined quicklime or anhydrous calcium chloride, to absorb water vapor. Color changing silica should preferably be used to indicate when it should be refreshed. Indication gels typically change from blue to pink, as they absorb moisture but other colors may be used.

The substance needing desiccation is put in the **upper compartment**, usually on a glazed, perforated ceramic plate. Its upper compartment also has a ground-glass rim, which must be greased with a thin layer of vacuum grease, petroleum jelly or other lubricant to ensure an airtight seal with the lid.

In order to prevent damage to a desiccator the lid should be carefully slided on and off, instead of directly placing it on to the base.

Lid of some of the desiccators may also have a stopcock, which helps in the evacuation of the desiccator. Such desiccators are also called **vacuum desiccators**. When a vacuum is to be applied, it is a common practice to criss-cross the vacuum desiccator with tape, or to place it behind a screen, to minimize damage or injury caused by an implosion. To maintain a good seal, vacuum grease is usually applied to the flanges.

AUTOMATIC PIPETTES

These are not truly the glass apparatus but are hand-held mechanical pipettes (push button pipettes) with a disposable tip. Both, the pipette and tips are made of PVC material. The disposable plastic tip and a push button are situated at the opposite end and are operated by the thumb (Fig. 13.30).

- These pipettes operate by piston-driven air displacement. A vacuum is generated by the vertical travel of a metal or ceramic piston within an airtight sleeve. As the piston moves upward, driven by the depression of the plunger, a vacuum is created in the space left vacant by the piston. The liquid around the tip moves into this vacuum (along with the air in the tip), transported and released as necessary.
- Liquid is drawn into, and dispensed from, a disposable pipette tip that is changed after each transfer. Depressing the tip ejector button removes the tip, that is cast-off without being handled by the operator and disposed off safely in an appropriate container.
- These pipettes are capable of being very precise and accurate. However, since they rely on air displacement, they are subject to inaccuracies caused by the changing environment, particularly, temperature and user technique. For these reasons, these must be carefully maintained and calibrated, and users must be trained to exercise correct and consistent technique.

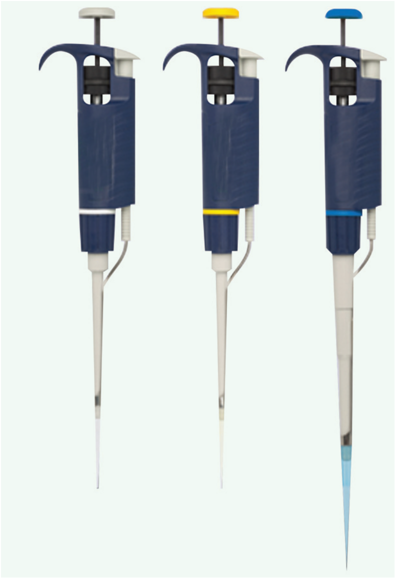


Fig. 13.30: Automatic pipettes

Types of Automatic Pipettes

They are available in volume range from 5 µL to 1.0 mL, both in the fixed volume and variable volumes.

Fixed Volume Pipette

The fixed volume pipette, as the name suggests, delivers a fixed volume only and that cannot be changed. As there are less moving parts, the mechanism is less complex, resulting in more accurate volume measurement.

Pipette with a Variable Volume

In a pipette with a variable volume, **adjustable volume pipette**, the volume of the liquid contained in the tip is variable. These types of pipettes can be set to a certain volume within its operational range, like 10–100 µL, which can be changed via a dial or other mechanism, depending on the model. Some pipettes also include a small window which displays the currently selected volume.

For optimal usage, every pipette supplier offers a broad range of different capacities. A small volume range of a pipette like 10–100 µL results in a much higher accuracy than a broad range from 0.1–1000 µL per pipette. These limits are indeed the limits and overwinding these limits would result in damage of the pipetting system.

Multichannel Electronic Pipettes

Depending on the number of pistons in a pipette, there are also multichannel pipettes, for manual high throughput applications, like filling up a 96-well microtitre plate. Instead of handling well by well, a row of 8 wells can be handled in parallel as this type of pipette has 8 pistons in parallel (Fig. 13.31).

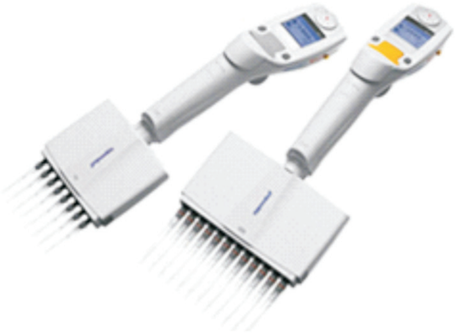


Fig. 13.31: Multichannel electronic pipettes

TIPS FOR AUTOMATIC PIPETTES

Automatic pipettes require **disposable tips** that come in contact with the fluid. The tips are plastic made tools for single use. In general, they are made of polypropylene. Depending on the size of the pipette, the user needs specific tip sizes like 10 µL, 100 µL, 200 µL, 1000 µL, or other nonstandard sizes such as 5 mL or 10 mL (Fig. 13.32).

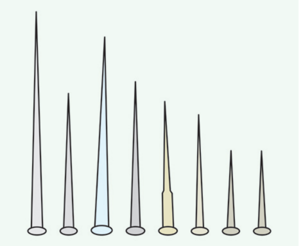


Fig. 13.32: Tips for automatic pipettes

The three standard sizes of disposable tips have different colors, a color code for easy spotting, like:

- Natural (clear) for low volumes (0.1–10 µL)
- Yellow (10–200 µL)
- Blue (100–1000 µL).

The corresponding pipette has the same color code, printed on the pipette.

The plastic pipette tips are designed for aqueous solutions, and are not recommended for use with organic solvents that may dissolve the plastics of the tips or even the pipettes.

PRECAUTIONS USING GLASS APPARATUS

- Use glassware that is without defect and has smooth edges. It should have no cracks, chips or scratches.
- Never set hot glassware on cold surfaces or in any other way that changes its temperature suddenly. It will break, if cold water is poured into a hot beaker.
- Never carry a flask by its neck.
- Never carry a beaker by its side.
- Always use two hands for carrying any glassware (position one hand under the glass for support).
- Appropriate glove should be worn when there is a risk of breakage, e.g. inserting a glass rod, chemical contamination, or thermal hazard.
- Avoid physical stress to the glassware. If necessary, stabilize it by using clamps and platforms to relieve pressure.
- Avoid overtightening clamp. Further, neck clamps should not be used as sole support for vessels larger than 500 mL.
- Ground glass joints are crafted for a perfect fit and may stick sometimes. Never force a joint free. It can cause the glass to shatter. Lubricate surfaces or use a Teflon sleeve.
- Never heat or cool glassware unless it is designed for those processes. Round bottom flasks are best for boiling liquids.

- When storing glassware, remember to keep it away from shelf edges. Place glassware toward the back of benches or hoods.
- Fume hoods and biosafety cabinets should **not** be used for storage of glassware.
- Do not let instruments roll around in drawers, use drawer pads.
- A laboratory worker should always be aware of the fact that glassware is likely to break, if handled roughly. Importance of handling glass apparatus, in the proper manner, cannot be over emphasized. While setting up an apparatus, it is necessary to ensure that all pieces are clamped and supported properly, different components may fall apart even with the smallest bumping and endanger both, the experiment and the worker.
- When cutting tubing or rod, make a simple cut at the point of cut with a triangular file or cutter. Place cloth over the glassware to protect hands. Then hold the tube or rod with the thumbs together, opposite the cut, and bend the material towards the body. A clean, right angle break will almost always be obtained but the cloth should always be used to guard against an odd accident.
- All glass edges should be fire polished, in a Bunsen flame, so as to round off the sharp corners, especially in case of glass tubing, which is to be pushed through a rubber bung.
- When glass containers have been used, they should be cleaned by the person who has used them. This ensures that other workers will not be endangered by small amounts of any harmful material remaining in the container. It is absolutely necessary that apparatus handed over for glass blowing has been cleaned thoroughly.
- Test tubes should be held properly with test tube holder while heating with the mouth pointing away from others and oneself, and should not be more than half full while being heated. Pipettes should not be left so that their ends protrude across the front edge of bench. While pipetting, always use pipette fillers (propipettes).
- Wash bottles or Winchester bottles made of colorless glass should not be left on benches where direct sunlight may fall, because they may act as lenses and focus sunlight on certain spots, and cause fire. Such instances have been reported in literature. Also, the window area should be free of any apparatus/bottles.
- The laboratory floor should be kept free of any glass apparatus or glass bottles containing chemicals.

HEATING AND COOLING

- When heating glassware, make sure to use only glassware made of borosilicate glass. Common glass can break, explode or shatter very easily when subjected to heat shock.
- Heating or cooling of most glassware must be done carefully because thermal expansion in one portion of the glass but not on adjacent portion may put too much mechanical stress on the surface and cause it to fracture.
- Heating of glassware should be slowed using an insulating material, such as metal foil or wool, or specialized equipment, such as heated baths, heating mantles or laboratory grade hot plates, to avoid fracturing. Never set hot glass on a cold bench top.
- Glassware should be vented when heating, to avoid over pressurization and possible explosion. Keep stoppers loose when autoclaving or during procedures that require heating.
- Never heat glassware that is etched, cracked, chipped, nicked or scratched.
- Glassware with thick walls, e.g. bottles and jars, should never be heated over a direct flame. Additionally, do not heat glassware directly on electrical heating elements.
- Do not look down into a vessel being heated.
- Cool all glassware **slowly** to prevent breakage, unless using specifically designed glassware.
- When using a Bunsen burner, the flame should touch the glass below the liquid level. A ceramic centered wire gauze will diffuse the burner flame to provide more even heat.
- Always use hotplates that are larger than the bottom of the vessel, being heated. When using a hot/stir plate, ensure that only the necessary settings are activated, i.e. if you do not intend to heat, ensure that the hot plate is **not** turned on.
- Use care when removing glassware from ultra-low temperature freezers (–70° to –150°C), to prevent thermal shock and cracking. For best results, immediately rinse the entire bottle under cold, running water, until thawing begins.
- Never place bottles directly from the freezer into warm water baths.

CLEANING AND CARE

Good lab technique necessitates the use of clean glassware. Glass must be physically and chemically clean and, in many cases, sterile. There are several methods of cleaning laboratory glassware. Most of the time, these methods are tried in this order:

- New glassware should be washed before use to remove any residue or loose particles. Clean immediately after use. The longer glassware sits, the harder it is to clean.
- A detergent solution may be used to soak glassware. This removes grease and loosens most contamination. Use laboratory grade detergents for cleaning glassware.
- Chromic acid (dichromate/sulfuric acid mixture) should never be used to clean glassware.
- Scrubbing with a brush or scouring pad is a mechanical means of removing gross contamination and large particles. When using brushes, make sure the metal part of the brush does not scratch the glass. Never use worn out cleaning brushes; they can scratch or abrade the glass.
- Solvents, such as mild acids, known to dissolve a specific contamination may be used to remove trace quantities.
- Acetone may be used for a final rinse of sensitive or urgently needed glassware as the solvent is miscible with water, and helps dilute and wash away remaining water from the glassware.
- For special type of precipitates, aqua regia (concentrate HCl : HNO_3 at 3 : 1 ratio) is used.
- When cleaning pipettes, place pipettes, tips down, into a cylinder or tall jar of water or appropriate disinfectant (e.g. for biologically contaminated tips). A pad of cotton or glass wool at the bottom will help prevent breakage of the tips. Ensure the water or disinfectant level is high enough to immerse the pipettes.
- Be sure to rinse glassware well.
- When drying glassware, place articles on towels, lined basket or slip-resistant pads. Be sure to place away from the edge of the bench. Large containers may be hung on pegs to dry.
- Glassware can also be dried by suspending it upside down to drip dry on racks, which may include a hot air fan to blow the internals dry.
- Another alternative is to place the glassware under vacuum, lowering the boiling point of the remaining volatiles.

CALIBRATION OF PIPETTES

Standardization of pipette is essential for accurate transfer of the specimen and can be easily done, e.g. if we are standardizing 1 mL of volumetric pipette, the following steps are to be undertaken:

- Weigh an empty beaker. Say the weight is x g
- Measure 1 mL of DW with the pipette + $x = y$ g
- $y - x$ = Weight of 1 mL of distilled water
- Repeat the above three steps at least two more times and calculate the mean.
- Calculate D.

D = Average expected weight – Average weight of 1 mL of DW

Average expected weight (EW) can be seen from the table showing weight in grams of 1 mL of DW at different temperatures.

Temp (°C)	20	25	26	27	28	29	30
EW	1.0	0.9989	0.9987	0.9984	0.9982	0.9979	0.9976

Now calculate % deviation error by the formula

$$\% \text{ Deviation error} = \frac{D}{EW} \times 100$$

It should not be greater than ±5%

Graduated Pipettes

Graduated pipettes are calibrated and marked with graduation lines that allow the measurement of various volumes. Graduated pipettes made of glass can be washed and reused. The pipettes should be plugged with cotton on the top and sterilized before use to minimize contamination of fluids being measured. Disposable plastic graduated pipettes are also available and are useful for pipetting toxic substances.

Micropipettes

Micropipettes are also available, which have plastic disposable tips and a digital display of volume. Micropipettes have three positions. Fit the tip to the end of the shaft. Press down and twist slightly to ensure an airtight seal.

- Hold the pipette in a vertical position.
- Depress the plunger to the first stop. Air equal to the volume of the setting say 100 μL is displayed.
- Immerse the tip into the liquid.
- Release the plunger back to the rest position. Let the liquid be sucked up in the tip.
- The volume of liquid in the tip will equal the volume of the setting of the micropipette. There should be no air bubbles in the pipette.
- While using multichannel pipettes volume should be same in all the tips.

Volumetric Pipettes

These are not graduated but designed specifically with a central bulb to deliver a specific quantity of the specimen. These pipettes are very accurate for pipetting solutions. The pipettes are marked as:

- T.C. (to contain): It is calibrated to contain a specified volume of liquid and the liquid is emptied by thorough rinsing of the pipette with the diluent in the receiving container.
- T.D. (to deliver): It is not to be rinsed after delivery of the liquid.

PRACTICAL EXERCISES

1. Identification of glassware and apparatus.
2. Use of pipettes and automatic pipettes.
3. Cleaning of laboratory glassware.
4. Calibration of automatic pipettes.

Important Questions

1. Describe various types of glass used for making glass apparatus.
2. Describe commonly used glass apparatus in a clinical chemistry laboratory.
3. Differentiate between:
    i. Conical flask and volumetric flask
    ii. Mohr type pipette and serological pipette
4. Write notes on:
    i. Vacuum desiccator
    ii. Cleaning and care of glass apparatus
    iii. Automatic pipettes
    iv. Calibration of pipettes

Chapter

14

Electrical Apparatus

In the laboratory, there is the potential for workers to be exposed to electrical hazards, including electric shock, electrocution, fire and explosion. Damaged electrical cord can lead to possible shock or electrocution. A flexible electrical cord may be damaged by door or window edges, by staples and fastenings, by equipment rolling over it, or simply by aging. The potential for possible electrocution or electric shock, or contact with electrical hazards can also be a result of faulty electrical equipment/instrumentation or wiring, damaged receptacles and connectors, and unsafe work practices.

Proper use and maintenance of laboratory equipment are essential to laboratory safety. Many laboratory accidents can be attributed to improperly maintained or improperly used laboratory equipment.

GENERAL PRECAUTIONS WHILE USING ELECTRICAL APPARATUS

- Ensure that **installation**, **modification** and **repair** of analytical equipment are carried out **by the authorized service personnel only**.
- **Read** and understand **manufacturer's instructions/manual** before using the equipment. The manual contains details of hazards and safety precautions to be taken during installation, operation and maintenance. The operating manuals of equipment must be located with the equipment or in a location that is easily accessible to all laboratory personnel.
- A **schedule for maintenance** and inspection of equipment must be prepared as per manufacturer's instruction and must be adhered to.
- **Safety devices** on laboratory equipment must never be disabled or altered.
- **Laboratory personnel must be trained** on the proper use of laboratory equipment prior to using the equipment. New users must carry out the operation under guidance of seniors.
- **Treat all electrical devices as if they are live** or energized. Disconnect the power source before servicing or repairing electrical equipment.
- Use only **tools** and **equipment with nonconducting handles** when working on electrical devices. Never use metallic pencils or rulers, or wear rings or metal watchbands when working with electrical equipment.
- When it is necessary to handle equipment that is plugged in, be sure hands are dry and, when possible, **wear nonconductive gloves**, **protective clothes** and shoes with insulated soles. If it is safe to do so, work with only one hand. This precaution reduces the likelihood of accidents that result in current passing through the chest cavity.
- **Keep work areas dry** when working with electrical apparatus. If water or a chemical is spilled on the equipment, shut off power at the main switch and unplug the equipment.
- **Minimize the use of electrical equipment in cold room** or other areas where condensation is likely to occur. If equipment must be used in such areas, mount the equipment on a wall or vertical panel. Switch off the equipment at the end of the operation and when not in use.
- **Source of power must be at an easily accessible location** so that it can be switched off quickly in case of emergency.
- **All electrical equipment should be grounded through three-phase plugs**.
- Electric wires of equipment must not be laid along the passage where it can be a trip hazard. Same must be kept away from hot surfaces which can cause damage to the insulation of the wire.

- **Extension cords should only be used when necessary and only temporarily**. You should always plug equipment directly into a permanent outlet only. Avoid any cord running through walls, up through the ceiling or down somewhere else.
- **Test all electrical apparatus, periodically** for voltage leaks.
- Inspect all electrical cords for defects and damage.
- Test all electrical receptacles and emergency cut-off switches for correct wiring and functioning.
- **Wear lab coats** and **safety glasses** for all procedures. Loose clothing and jewelry, that can get entangled, must not be used. Confine long hair while working in the laboratory.
- **Do not store** highly flammable liquids **near electrical equipment**. Sparks may result in explosion in area where flammable liquids are used. Therefore, proper grounding of the equipment and containers should be done.
- **Maintain a work space clear of extraneous material**, such as books, papers and clothes.
- **Equipment producing a tingle should be disconnected** and reported promptly for repair.

CENTRIFUGE MACHINES

A centrifuge machine is a piece of equipment that puts an object in rotation around a fixed axis (spins it in a circle), applying a potentially strong force perpendicular to the axis of spin (outward). Thus, a centrifuge machine can be described as a machine with a rapidly rotating container that applies centrifugal force to its contents (Fig. 14.1).

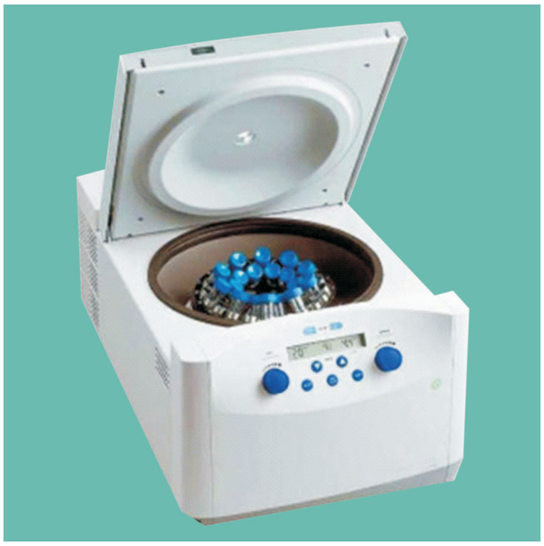


Fig. 14.1: A centrifuge machine

The centrifuge works using the sedimentation principle, where the centripetal acceleration causes denser substances and particles to move outward in the radial direction. At the same time, objects that are less dense are displaced and move to the centre.

Types of Centrifuge Machines

There are multiple types of centrifuges which can be classified by intended use or by rotor design.

Intended Use

- **Laboratory centrifuges** are general purpose instruments of several types with distinct, but overlapping, capabilities. These include clinical centrifuges, superspeed centrifuges and preparative ultracentrifuges.
- **Analytical ultracentrifuges** are designed to perform sedimentation analysis of macromolecules using the principle devised by Theodor Svedberg.
- **Hematocrit centrifuges** are used to measure the volume percentage of red blood cells in whole blood.

Rotor Design

- **Fixed angle centrifuges** are designed to hold the sample containers at a constant angle relative to the central axis.
- **Swing head (or swinging bucket) centrifuges**, in contrast to fixed angle centrifuges, have a hinge where the sample containers are attached to the central rotor. This allows all of the samples to swing outwards as the centrifuge is spun.
- **Continuous tubular centrifuges** do not have individual sample vessels and are used for high volume applications.

Laboratory Centrifuges

In a laboratory centrifuge, that uses sample tubes, the radial acceleration causes denser particles to settle to the bottom of the tube while low density substances rise to the top.

A wide variety of laboratory centrifuges are used in chemistry, biology, biochemistry and clinical medicine for isolating, and separating suspensions and immiscible liquids. They vary widely in speed, capacity, temperature control and other characteristics. Laboratory centrifuges often can accept a range of different fixed angle and swing bucket rotors to carry different number of centrifuge tubes and rated for specific maximum speed. Controls vary from simple electrical timers to programmable models to control acceleration and deceleration rates, running speed and temperature regimes.

Ultracentrifuges spin the rotors under vacuum, eliminating air resistance and enabling exact temperature control.

Because of the heat generated by air friction and the frequent necessity of maintaining samples at a given temperature, many types of laboratory centrifuges are refrigerated and temperature regulated.

Centrifuge Tubes

Centrifuge tubes are precision made, high strength tubes of glass or plastic, made to fit exactly in rotor cavities. They may vary in capacity from 50 mL down to much smaller capacities used in microcentrifuges, extensively in molecular biology laboratories. Microcentrifuges, typically, accommodate disposable plastic microcentrifuge tubes with capacities from 250 µL to 2.0 mL.

Glass centrifuge tubes can be used with most solvents but tend to be more expensive. They can be cleaned like other laboratory glassware and can be sterilized by autoclaving. They must be handled with care since small scratches can cause failure under the strong forces imposed during a run. Glass tubes are inserted into soft rubber sleeves, to cushion them during run.

Plastic centrifuge tubes, especially tend to be less expensive and, with care, can be just as durable as glass. Water is preferred when plastic centrifuge tubes are used. They are more difficult to clean thoroughly and are usually inexpensive enough to be considered disposable.

Disposable plastic (Eppendorf tubes) of 0.5 mL to 2 mL are commonly used in microcentrifuges. They are moulded from a flexible transparent plastic, similar to polythene and are semi-conical in shape with integral, hinged sealing caps.

Larger samples are spun using centrifuge bottles, which range in capacity from 250 to 1000 milliliters. Although some are made of heavy glass, centrifuge bottles are usually made of shatter proof plastics, such as polypropylene or polycarbonate. Sealing closures may be used for added leak proof assurance.

Safety Measure while using Centrifuge Machines

- **Standard operating procedures** should be placed near the centrifuge machine. Users should operate the centrifuge according to the instructions in the manual. All operators should be trained on proper operating procedures before operating the centrifuge. Where necessary, logbook must be filled in.
- User must always **ensure that power cable is in good condition** and that no wire is exposed.
- **As centrifuge rotor** is subjected to a high mechanical stress at rotation speeds, accidents result from improper loading or balancing of the rotor. This causes them to dislodge during spinning, resulting in serious damage and personal injury. **Before use, check the rotor, lids and seals are clean and not damaged.**
- **Centrifuge rotors should never be touched while the rotors are moving**, because a spinning rotor can cause serious injury. If the centrifuge has a lid, make sure that it is closed during operation. Do not try to stop a centrifuge by grabbing it. Make sure that the centrifuge machine is completely stopped, before removing test tubes from it. Do not run a rotor beyond its maximum rated speed. Do not open lid during or immediately after operation. Do not interfere with the interlock safety device.
- **Check** glass and plastic **centrifuge tubes for stress lines**, **hairline cracks** and **chipped rims**, **before use**. Use unbreakable tubes whenever possible. Use caps or stoppers on centrifuge tubes, if provided.
- **Do not overfill the centrifuge tubes**. A buildup of chemicals from spillages may cause tube to jam in rotor or corrosion, which could lead to rotor failure.
- **Position test tubes opposite to each other, with the same weight in each tube**, to balance the centrifuge, i.e. balance the load properly. Use water filled tubes, if necessary for balance. Small differences in mass of the load can result in a large force imbalance when the rotor is at high speed. This force imbalance strains the spindle and may result in damage to centrifuge and even may cause personal injury. An unbalanced centrifuge can also walk off the table.
- Always **use sealed safety cups or sealed rotors with O-rings when working with infectious agents**. Wipe exterior of tubes or bottles with disinfectant prior to loading. Open rotor in biological safety cabinet when working with infectious agents. Decontaminate safety carriers or rotors and centrifuge interior after each use.
- Make sure that centrifuge operates vibration free, even at the top speed. **Stop the centrifuge immediately, if an unusual condition** (noise or vibration) **begins**.
- Keep hair, loose clothing and body parts away from the centrifuge while it is operating.
- Never leave a running centrifuge unattended.

WATER BATHS

A water bath is a laboratory equipment made from a container filled with heated water (Fig. 14.2).

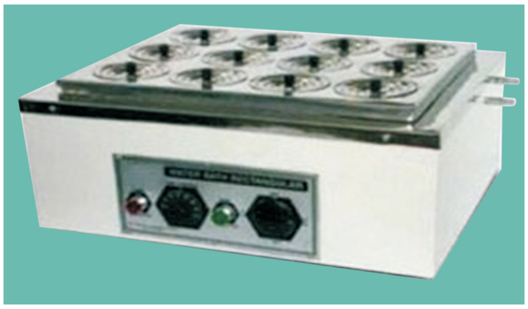


Fig. 14.2: Water bath

It is used to incubate samples in water at a constant temperature, over a long period of time. All water baths have a digital or an analogue interface to allow users to set a desired temperature. Utilizations include warming of reagents, melting of substances or incubation of cell cultures. It is also used to enable certain chemical reactions to occur at high temperature. Water bath is a preferred heat source for heating flammable chemicals instead of an open flame, to prevent ignition. Different types of water baths are used depending on their application. Water baths can be used up to 99.9°C. When the required temperature is above 100°C, alternative methods of heating, such as oil bath, silicone bath or sand bath, should be used.

Types of Water Baths

Circulating Water Baths

Circulating water baths (also called stirrers) are ideal for applications when temperature uniformity and consistency are critical, such as enzymatic and serologic experiments. Water is thoroughly circulated throughout the bath, resulting in a more uniform temperature.

Noncirculating Water Baths

This type of water bath relies primarily on convention, instead of water being uniformly heated. Therefore, it is less accurate in terms of temperature control. In addition, there are add-ons that provide stirring to noncirculating water baths, to create more uniform heat transfer.

Shaking Water Baths

This type of water baths have extra control for shaking, which moves liquids around. Shaking feature can be turned on or off. In microbiological practices, constant shaking allows liquid grown cell cultures to constantly mix with the air.

A water bath is a laboratory equipment made from a container filled with heated water. It is used to incubate samples in water at a constant temperature, over a long period of time. All water baths have a digital or an analogue interface to allow users to set a desired temperature. Water bath is a preferred heat source for heating flammable chemicals instead of an open flame to prevent ignition. Different types of water baths are used, depending on the application, up to any temperature below 100°C.

Safety Measure while Using Water Baths

- It is not recommended to use water bath with moisture sensitive or pyrophoric reactions. It should be set on a steady surface, away from a flammable material.
- Water level should be regularly monitored. If possible, fill it with distilled water, which will help to prevent salt from depositing on the heater.
- Unplug the unit before filling or emptying water.
- The cover should be closed to prevent evaporation and to help reaching, required, high temperature.
- Raise the temperature to 90°C or higher, once a week for half an hour, for the purpose of decontamination.
- Clean regularly. A disinfectant, such as a phenolic detergent, can be added to the water, to prevent growth of the organisms. Avoid using sodium azide for this purpose, as sodium azide forms explosive compounds with some metals.
- Markers tend to come off easily in water baths, so use water resistant markings.
- If application involves liquids that give off fumes, it is recommended to operate water bath in fume hood or in a well ventilated area.

HOT PLATES

A hot plate is a portable self-contained tabletop small appliance that features an electric heating element (Fig. 14.3).

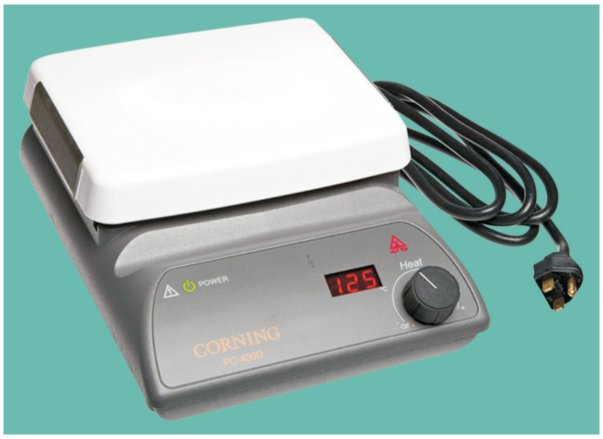


Fig. 14.3: Hot plate

A hot plate can be used as a stand alone appliance, but is often used as a substitute for the burner. In laboratory settings, hot plates are generally used to heat glassware or its contents. Typical operating temperature may vary from 100°C to 750°C. A hot plate can have a flat or round surface.

The following precautions must be taken while using a hot plate:

- Use a hot plate with a smooth, clean surface.
- Hot plates appear exactly the same whether hot or at room temperature. So, always assume that they are hot and act accordingly.
- Care must always be taken to ensure that there is sufficient clearance between the appliance location and combustible material above them. In addition, a hot plate should not be located in a place where the heat control of the appliance is accessible to children and others who are not familiar with its operation.
- The cord of the hot plate should be checked periodically, for frays and faults. Any hot plate with faulty wiring should not be used. It should be repaired or replaced immediately.
- Ensure that electrical cord of the hot plate does not come in contact with the heating surface. Keep the electrical cord of a hot plate away from water.
- Controls on the hotplate must be properly labeled to easily distinguish between the stirrer and the temperature controls.
- Limit use of hotplate for flammable materials as they can pose an electrical spark hazard.
- Laboratory workers should be warned of the spark hazard associated with older hot plates.

WEIGHING BALANCES

These are mainly used to weigh the chemicals accurately for the preparation of analytical reagents.

Types of Weighing Balances

Various types of balances are as shown below.

1. Single Pan Balance

These are electronically operated and work on the principle of substitution of weights (Fig. 14.4).

Characteristic features of these balances are:

- High accuracy (0.1–0.01 mg)
- High sensitivity
- Easy handling
- Quick weighing
- Easy readable rotary scale.

These are the preferred balances nowadays.

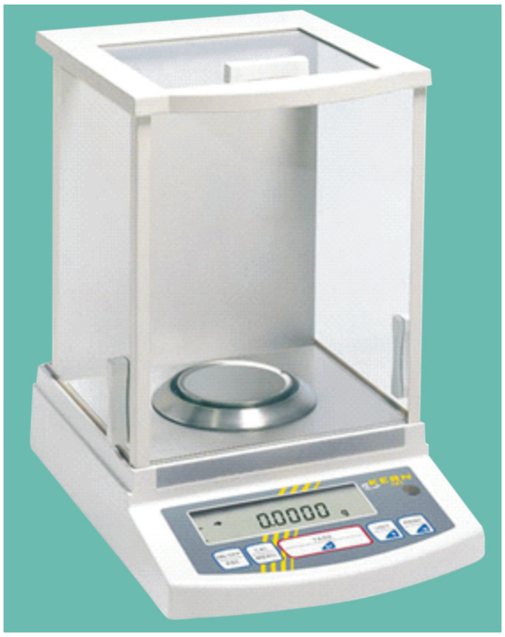


Fig. 14.4: Single pan electric balance

2. Double Pan Balance

This is most frequently used balance, which is also quite accurate, if the weights used are of standard quality. These are mechanical balances in which chemicals can be weighed from 100 mg to few grams. However, they are cumbersome. Weight of the container has also to be taken into account.

3. Electric Balance

In the electronic balance, initially the system is in null position when no weight is placed on the single pan. When some chemical is placed on the pan, the null position gets disturbed. An electromagnetic force is used to return the balance beam to its null position. The restoring force, which is applied with the help of a torque motor, is proportional to the weight on the pan. The current required to produce the force is displayed digitally, which is equivalent to the mass kept on the pan.

Safety Measures while Using Weighing Balances

- All the balances have to be handled gently and placed on a levelled hard surface away from wind, radiation, vibrations, dust, heat, etc.
- The beam of the balance should be arrested when not in use.

- Weights should not be allowed to lie on the pans
- The balance should not be loaded with the weight greater than the maximum, it is constructed to weigh.
- The substance to be weighed, should not be hot
- The cabinet of the balance should be kept close when the reading is being taken.
- Cover the balance when not in use
- Put the chemical to be weighed on a butter paper and not directly on the pan.
- Clean the balance after use
- Remove any powder or dust with the help of a tissue and never blow it, as it could transport the spilled sample material inside the balance.
- For removal of sticky substances use a damp lint-free cloth and mild solvent like 70% ethanol.
- Remove parts for cleaning wherever possible as per instructions in the manual.

PRACTICAL EXERCISES

1. Identification of equipments.
2. Care and handling of centrifuge machines.
3. Care and handling of water baths.

Important Questions

1. Describe general precautions while using various electrical apparatuses.
2. What is a centrifuge machine? Describe various types of centrifuge machines. What safety measures should be observed while using a centrifuge machine?
3. Write notes on:
   i. Various types of water baths
   ii. Hot plate
   iii. Various types of weighing balances
   iv. Cleaning and care of weighing balance

Chapter

15

Collection and Handling of Blood Specimen

Clinical chemistry, also known as **chemical pathology**, **clinical biochemistry** or **medical biochemistry**, is the area which is generally concerned with the analysis of body fluids. The discipline originated in the late 19th century with the use of simple chemical tests for various components of blood and urine. Subsequent to this, other techniques were applied, including the use and measurement of enzyme activities, spectrophotometry, electrophoresis and immunoassays.

Order Form/Requisition Slip

A requisition form must accompany each sample submitted to the laboratory. The requisition form must contain proper information in order to process the specimen. The essential elements of the requisition form are:

- Patient's name.
- OPD/CR number.
- Patient's age and sex.
- Requesting physician's signature and stamp.
- Source of specimen: This information must be given when requesting microbiology, cytology, fluid analysis or other testing, where analysis and reporting is site specific.
- Date and time of collection.
- Name/list of the test(s) requested.

Biochemical tests may be performed on any kind of body fluid but mostly blood, plasma or serum is used for this purpose. However, the type of test required dictates, what type of sample is to be used?

BLOOD SPECIMEN

WHOLE BLOOD, SERUM OR PLASMA

Historically, serum is preferred for determining extracellular concentration of blood constituents. These days, however, even plasma is used for many, but not all, laboratory investigations. It is imperative that the *in vivo* state of a constituent should not change even after withdrawal of the sample from a patient, to obtain valid results.

Whole Blood

Whole blood is a body fluid that delivers necessary substances, such as nutrients and oxygen to the cells and transports metabolic waste products away from the cell.

- Blood is composed of various types of cells suspended in plasma. These include red blood cells (also called RBCs or erythrocytes) and white blood cells, including leukocytes and platelets.
- In a venous, arterial or capillary blood sample, concentration and properties of the various cellular and extracellular constituents remain relatively unaltered, when compared with their *in vivo* state.
- Addition of anticoagulant, *in vitro*, stabilizes the constituents in the whole blood sample, for a certain period of time.

Serum

Serum is undiluted **yellow watery part of blood**, which is left after blood has been allowed to clot and all the blood cells have been removed. This is, generally, done by centrifugation, which packs blood cells and platelets to the bottom of the centrifuge tube, leaving liquid (serum) fraction, resting above the packed cells. For this purpose:

- Blood is collected in a tube **without any anticoagulant** and **allowed to clot**.
- Thereafter, leave the tube at room temperature for at least 30 minutes, for the separation of the serum.
- Serum is obtained after a clot is formed.

- Centrifuge the sample to obtain a clear supernatant (serum).

Plasma

Plasma is **cell-free supernatant of blood containing an anticoagulant**. It is obtained after centrifugation of the blood. Plasma constitutes about 55% of blood fluid. It is mostly water (92% by volume) and contains dissipated proteins, glucose, mineral ions, hormones and carbon dioxide. From the same volume of blood, about 15 to 20% more volume of plasma is obtained than serum. For this purpose:

- **Blood is collected in anticoagulant tubes** and **mixed**, by gently inverting the tube, several times. This, in turn, ensures even distribution of anticoagulant with the specimen.
- Only a recommended quantity of the anticoagulant should be added, wherever required, to avoid any error in result.

ANTICOAGULANTS

Anticoagulants are the additives, which inhibit blood and/or plasma from clotting, ensuring that the constituents to be measured are not significantly altered, prior to the analytical process. Anticoagulation occurs by binding of the calcium ions (such as by EDTA or citrate) or by inhibiting the thrombin activity (such as by heparin).

Commonly used Anticoagulants

Commonly used anticoagulants, which may be used in sample collection as mentioned below:

EDTA

- EDTA (ethylenediaminetetra-acetic acid) dipotassium ($EDTA.K_2$), tripotassium ($EDTA.K_3$) or disodium ($EDTA.Na_2$) salt, available in the powder form, may be used in a concentration of 1.2 to 2.0 mg/mL of blood.
- It strongly and irreversibly chelates calcium ions and prevents blood from clotting.
- EDTA (especially, dipotassium salt) interferes in some of the tests. Under these situations, heparin should be used as an alternative.
- Tubes with liquid EDTA reduce risk of hemolysis, which, sometimes, may be seen by using EDTA powder.

Citrate

- Trisodium citrate or acid-citrate-dextrose, with 0.10 to 0.136 mol/L (3–4%) citric acid (as buffered-citrate, pH 5.5 to 5.6) is used in the liquid form, in tubes for coagulation tests as well as in blood transfusion bags.
- Correct proportion of the anticoagulant is important to avoid dilution of the blood. Generally, a mixture of one part of citrate with nine parts of the blood is recommended for coagulation tests while one part of the citrate is mixed with four parts of the blood to determine erythrocyte sedimentation rate (ESR).
- Citrate binds calcium but not as strongly as EDTA.

Oxalate

Its mechanism is similar to that of citrate. It is used in fluoride-oxalate tubes, for use in blood collection for glucose and lactate determinations.

Heparin

About 12 to 30 IU/mL of the unfractionated sodium, lithium or ammonium salt of heparin is recommended. Calcium-titrated heparin, at a concentration of 8 to 12 IU/mL of blood, is recommended for the determination of ionized calcium.

Fluoride

For blood glucose determination fluoride is used as anticoagulant.

VACUTAINERS

A vacutainer, **blood collection tube**, is a sterile glass or plastic test tube with a colored rubber stopper creating a vacuum seal inside of the tube, thereby, facilitating the draw of a predetermined volume of blood. They are available with a safety engineered stopper and a variety of labelling options and draw volumes. Each tube is topped with a color-coded plastic or rubber cap. Tubes often include additives that mix with the blood when collected and the color of each tube's plastic cap indicates which additive it contains.

Principle of working of a vacutainer: Vacutainer needles are double ended, with one side being encased in a thin rubber coating for safety. When the needle is screwed into the translucent plastic needle holder, the rubber needle (inside the holder) and the exposed needle will be inserted into the vein. When a vacutainer tube is inserted into the holder, its rubber cap is punctured by the inner needle and the vacuum in the tube pulls blood through the needle into the tube. The filled tube is then removed and another can be inserted and filled the same way. The amount of air evacuated from the tube predetermines how much blood will fill the tube before blood stops flowing.

After the tube has been filled with blood, immediately invert the tube, containing additive, several times to prevent coagulation.

These tubes have an expiry, because over time, the vacuum is lost and blood will not be drawn into the tube when the needle punctures the cap.

Types of Vacutainers

- **Red-top tube (plain tube):** This tube is a plain vacutainer containing no anticoagulant and is used for collection of serum for selected chemistry tests as well as clotted blood for immunohematology.
- **Gold-top tube (serum gel tube):** This tube contains a clot activator and serum gel separator, and is used for various laboratory tests. Invert the tube to activate clotting, let it stand for 20 to 30 minutes before centrifuging for 10 minutes. If frozen serum is required, pour off serum into plastic vial and freeze. Do not freeze the vacutainer.
- **Green-top tube (sodium heparin tube):** This tube contains sodium heparin or lithium heparin and is used for collection of heparinized plasma or whole blood in clinical chemistry, e.g. urea and electrolyte determination. Sodium heparin collection tubes are preferred for peripheral blood or bone marrow for cytogenetic studies. Lithium heparin is considered suboptimal for cytogenetics.
- **Purple or lavender-top tube (K_2.EDTA tube):** EDTA is a strong anticoagulant and these tubes are used for most hematological procedures, such as complete blood counts (CBC). These can also be used for some blood bank procedures, such as blood type and screening. EDTA tubes are also preferred by most molecular laboratories for genetic (DNA or RNA) studies.
- **Yellow-top tube (ACD tube):** This tube contains sodium polyanethol sulfonate (SPS) used for blood culture specimens, or acid-citrate-dextrose (ACD) for drawing whole blood required for special tests for blood bank studies, HLA phenotyping and paternity testing.
- **Grey-top tube (potassium oxalate/sodium fluoride tube):** This tube contains potassium oxalate as an anticoagulant and sodium fluoride as a preservative. Fluoride prevents enzymes in the blood from working, by preventing glycolysis and is used to preserve glucose in whole blood.

Order of Draw

The term order of draw refers to the sequence in which tubes should be filled. Since the needle, which pierces the tubes, can carry additives from one tube to the next, the sequence is standardized to avoid any cross-contamination of additives, so that the laboratory results are not affected.

If using vacutainers, the order of collecting the blood should be:

1. Blood culture tube (yellow or yellow-black top)
2. Coagulation tube (light blue top).

   If just a routine coagulation assay is the only ordered test, then a single light blue top tube may be used. If there is a concern regarding contamination by tissue fluids or thromboplastin, one may draw a non-additive tube first and then the light blue top tube.
3. Nonadditive tube (red top).
4. Lastly, draw the tubes with different additives in the following order:
   - SST (red-gray or gold top), which contains a clot activator.
   - Sodium heparin (dark green top).
   - PST (light green top), which contains lithium heparin.
   - EDTA (lavender top).
   - ACDA or ACDB (pale yellow top), which contains acid-citrate-dextrose.
   - Oxalate/fluoride (light gray top)

Timing of Sample Collection

As we know, blood composition of various analytes is significantly altered after consuming food, thus, it is necessary to provide the patient, in advance, with the appropriate collection instructions, information on fasting or diet and medication restrictions, if required.

- In routine, **overnight fasting sample** (early morning, approximately 12 hours after the last ingestion of food) is recommended for determining concentration of common blood constituents, such as glucose, triglycerides, lipoprotein fractions, electrolytes, proteins, etc.
- For glucose alone, **fasting of four hours** is sufficient.
- Blood can be taken at **any time of the day** for some of the tests, such as for total cholesterol.

Position of the Subject

Position of the subject can also influence results of some of the analytes, thus, standardization of the position is necessary.

- It is, generally, recommended that blood sample should be drawn in a sitting position.
- The subject should remain in the sitting position for about 15 minutes, prior to blood collection. This (waiting period) will allow equilibration of concentration of most of the blood components.

Preparation of the Patient

- Exercise (muscular activity) has, both, transient as well as long lasting effects. Thus, creatine kinase (CK), aspartate aminotransferase (AST), lactate dehydrogenase (LDH) and platelet counts may increase.
- Stress may cause transient elevation in white blood cells (WBCs) and elevated adrenal hormone values (cortisol and catecholamines). Anxiety results in hyperventilation. It may cause acid–base imbalance and increase lactate concentration.
- Diurnal rhythms are body fluid and analyte fluctuations, during the day. For example, serum cortisol level is highest in early morning but is reduced in the afternoon. Serum iron levels tend to drop during the day. One must check timing of these variations for the desired collection point.
- Postural changes, supine to sitting, etc., are known to vary lab results of some of the analytes. Certain large molecules are not filtered into the tissue; therefore, they are more concentrated in the blood. Levels of several enzymes, proteins, lipids, iron and calcium are significantly increased with a change in position.
- Age, gender and pregnancy also have influence on laboratory results. Normal reference range is often noted according to age.

COLLECTION AND HANDLING OF BLOOD SAMPLE

Proper sample collection and handling is an integral part of obtaining a valid laboratory test results. Thus, a specimen must be:

- Obtained using **proper technique**
- Collected in the **proper container**
- **Correctly labeled** (in the presence of patient)
- **Promptly transported** to the laboratory, for analysis.

Withdrawl of Blood

There are many ways in which blood can be drawn from the vein. The method, however, may vary with age of the patient, equipment available and the tests required.

Various techniques of blood collection include:

- **Venipuncture:** Venipuncture using a needle and tube is the most common method for blood collection.
- **Vacutainer:** Blood collection can also be done with an evacuated tube system called vacutainer, which consists of a plastic hub, a hypodermic needle and a vacuum tube.
- **Lancet:** Blood may also be collected by puncturing the skin with a retractable lancet device.

VENIPUNCTURE

Venipuncture, **venopuncture** or **venepuncture** is a process of obtaining intravenous access for blood sampling of venous blood. This procedure is performed by a **phlebotomist** (professional who draws blood), nursing staff, medical laboratory technologists or medical practitioners. Though it is a complex procedure, requiring both knowledge and skill to perform, it is one of the most routinely performed invasive procedures carried out to obtain blood for diagnostic purposes as well as to monitor levels of blood components.

Requirements for Venipuncture

- **Vacutainers:** The tubes are designed to fill with a predetermined volume of blood, by vacuum. These are available in various sizes. Their rubber stoppers are color-coded, according to the additive, which the tube contains. Since these tubes have different additives or coatings, blood should never be poured from one tube to another (Fig. 15.1).

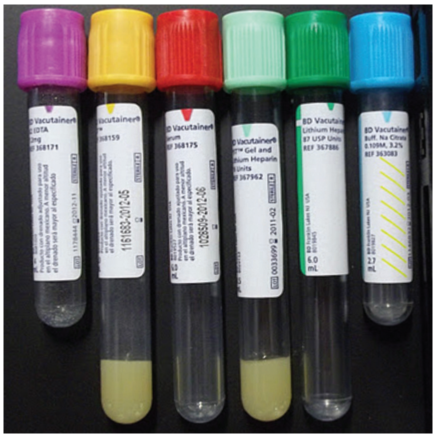


Fig. 15.1: Vacutainers

- **Needles:** Needles are available for evacuated systems and for use with a syringe, single draw or butterfly system. The gauge number indicates the bore size. Larger the gauge number, smaller is the needle bore.
- **Holder/adapter:** It is used with the evacuated collection system.
- **Tourniquet**
- **Alcohol wipes:** 70% isopropyl alcohol.
- **Povidone-iodine wipes/swabs:** These are used if blood is to be drawn for culture.

- **Gauze sponge:** It is required for application on the site from which the needle is withdrawn.
- **Adhesive bandage/tape:** It protects the venipuncture site after collection.
- **Needle disposal unit:** Needle should never be broken, bent, or recapped. It should be placed in a proper disposal unit, immediately after use.
- **Gloves:** Gloves made of latex, rubber, vinyl, etc., are worn to protect the patient and the phlebotomist.
- **Syringe:** It may be used in place of the evacuated collection tube, for special circumstances.

Use of Tourniquet

1. Prolonged venous occlusion can cause change in the concentration of blood constituents. Therefore, use of tourniquet should be minimized. If a tourniquet is used to search for a vein, it should be released before withdrawal of blood begins. In any case, use of a tourniquet should be limited to less than one minute.
2. Select a suitable site for venipuncture, by placing tourniquet 3 to 4 inches above the selected puncture site, on the patient.
3. Do not put tourniquet too tight and do not leave it on the patient for longer than 1 minute.
4. It should be wiped-off with alcohol and replaced frequently, because:

Prolonged use of tourniquet may have some undesirable effects, viz:

- Primary effect is hemoconcentration of nonfilterable elements (i.e. proteins). The hydrostatic pressure causes some water and filterable elements to leave the extracellular space.
- Significant increase can be found in total protein, aspartate aminotransferase (AST), total lipids, cholesterol and iron.
- Affects packed cell volume (PCV) and other cellular elements.
- Hemolysis may occur, with pseudohyperkalemia.

Site of Venipuncture

- Preferably blood should not be collected from the arm that is used for blood pressure measurement, i.e. blood should usually be drawn from the **left arm**.
- Blood sample should be taken from vein in the **antecubital fossa**.
- Before blood collection, the subject should **remove tight clothes**, which may constrict the upper arm.
- During blood collection, the **arm should rest on some supportive prop**.
- Though **large median cubital** and cephalic **veins** are the usual choice but basilic vein on the dorsum of the arm or dorsal hand veins are also acceptable.
- **Foot veins** are the last resort because of the higher probability of complications.
- Palpate and trace path of veins with the index finger. Arteries pulsate, are most elastic and have a thick wall. Thrombosed veins lack resilience, feel cord-like and roll easily.
- If superficial veins are not readily apparent, you can force blood into the vein by massaging the arm from wrist to elbow, tap the site with index and second finger, apply a warm, damp washcloth to the site for 5 minutes, or lower the extremity over the bedside, to allow the vein to fill.

Areas to Avoid when Choosing a Site for Blood Withdrawl

Certain areas should be avoided when choosing a site for blood withdrawl:

- **Extensive scars from burns and surgery**, since it is difficult to puncture the scar tissue and obtain a specimen from there.
- **The upper extremity on the side of a previous mastectomy**, because test results may be affected due to lymphedema.
- **Hematoma**, since it may cause erroneous test results. If another site is not available, collect specimen distal to the hematoma.
- **Intravenous therapy (IV)/blood transfusion**, since fluid may dilute the specimen. If possible, collect from the opposite arm. Otherwise, a sample may be drawn below the IV, by following these procedures:
  - Turn off the IV set for at least 2 minutes before venipuncture.
  - Apply tourniquet below the IV site.
  - Select a vein other than the one with the IV set.
  - Perform venipuncture, draw 5 mL of blood and discard it, before drawing specimen, in tubes, for testing.
- **Intravenous line**, since drawing blood from an intravenous line may introduce problem, the line must be flushed, first. When using a syringe inserted into the line, blood must be withdrawn slowly to avoid hemolysis.
- **Fistula or cannula** blood should not be drawn from an arm with a fistula or cannula without consulting the attending physician.
- Edematous extremities should be avoided, as tissue fluid accumulation alters test results.

Venipuncture Procedure

Several essential steps are required for successful blood collection:

1. A phlebotomist (professional who draws blood) must be a professional, who should be courteous and have understanding with the patient.
2. First step to blood collection is to positively identify the patient by two forms of identification, i.e. ask the patient to state and spell his/her name, and give his/her age. Check these against the requisition form.
3. Check the requisition form for requested tests, patient's information and special requirement, if any
4. Gather the tubes and supplies, that will be required for the withdrawl of blood.
5. Position the patient in a chair, or sitting or lying on a bed.
6. Wash hands.
7. Select a suitable site for venipuncture, by placing tourniquet 3 to 4 inches above the selected puncture site, on the patient. Do not put the tourniquet too tightly or leave it on the patient for longer than 1 minute.
8. Put on nonlatex gloves and palpate for the vein.
9. After the vein has been selected, clean the area in a circular motion, beginning at the site and working outwards. Allow the area to air dry. After the area is cleansed, it should not be touched or palpated again. If it is necessary to re-evaluate the site by palpation, the area should be re-cleansed before the venipuncture is performed.
10. Grasp patient's arm firmly, using thumb, to draw the skin taut and anchor the vein.
11. Swiftly insert the needle, through the skin, into lumen of vein. The needle should form 15 to 30° angle with the arm surface and avoid excess probing (Fig. 15.2).

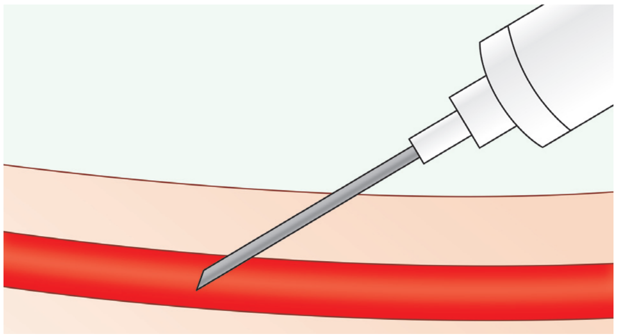


Fig. 15.2: Venipuncture

12. When the last tube is being filled, remove tourniquet.
13. Remove needle from patient's arm, using a swift backward motion.
14. Immediately place gauze on the puncture site.
15. Apply and hold adequate pressure to avoid formation of hematoma.
16. After holding pressure for 1–2 minutes, tape a fresh piece of gauze to the puncture site.
17. Dispose off the contaminated materials/supplies, in the designated containers.
18. Mix, and label all appropriate tubes at the patient's bedside.
19. Deliver specimens promptly to the laboratory.

Troubleshooting

If an Incomplete Collection or no Blood is Obtained

- Change the position of the needle. Move it forward (it may not be in the lumen) or move it backward (it may have penetrated too far; Fig. 15.3).

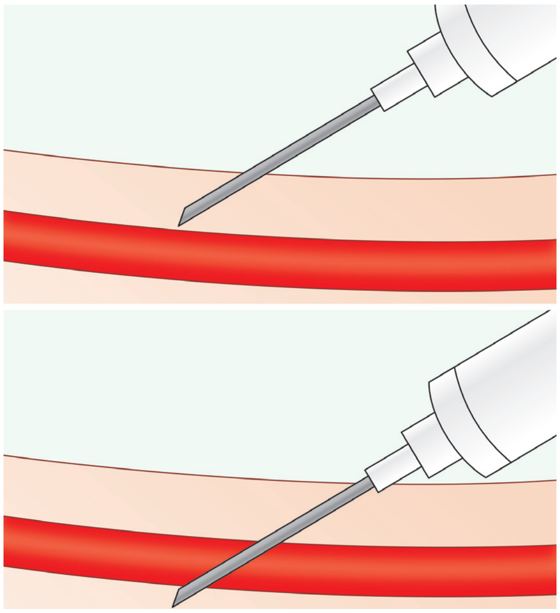


Fig. 15.3: Adjustment of position of the needle (above—by moving it forward, or below—by moving it backward)

- Adjust the angle (the bevel may be against the vein wall).
- Loosen the tourniquet. It may be obstructing blood flow.
- Try another tube. Use a smaller tube with less vacuum. There may be no vacuum in the tube being used.

- Re-anchor the vein. Veins sometimes roll away from the point of needle or puncture site.
- Ask the patient to make a fist and flex the arm, which helps engorge muscles to fill veins.
- Pre-warm region of the vein to reduce vasoconstriction and increase blood flow.
- Ask the patient to drink fluid, if dehydrated.

If Blood Stops Flowing

- The vein may have collapsed. Re-secure tourniquet to increase venous filling. If this is not successful, remove the needle, take care of the puncture site and redraw.
- The needle might have pulled out of the vein when switching tubes. Hold equipment firmly and place fingers against patient's arm, using the flange for leverage when withdrawing and inserting tubes.

Problems Other than an Incomplete Collection

- A hematoma may form under the skin adjacent to the puncture site. If so, release tourniquet immediately and withdraw the needle. Hematoma formation is a common problem in older patients.
- Apply firm pressure (Fig. 15.4).

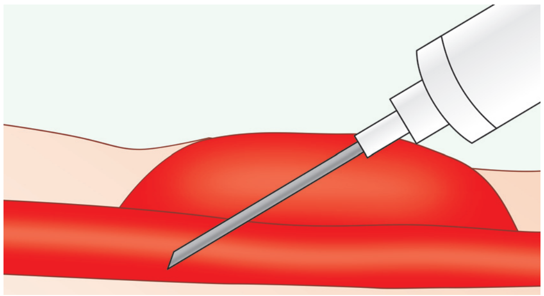


Fig. 15.4: Hematoma

Hemoconcentration

Hemoconcentration (an increased concentration of large molecules and formed elements in the blood) may be due to several factors:

- Prolonged tourniquet application (it should not be for more than 1 minute)
- Massage, squeezing or probing a site
- Long-term IV therapy
- Sclerosis or occluded vein

Fingerstick Procedure

- Follow the procedure as outlined above, i.e. identify the patient and the requisition form indicating the ordered test(s).
- Verify patient's condition, such as fasting, dietary restrictions, medications, timing, medical treatment, etc., since all the information is of concern and should be noted on the lab requisition slip.
- Position the patient who should either sit in a chair or lie down/sit in bed. Hyperextend the patient's arm.
- Best location for fingersticks is the 3rd (middle) and the 4th (ring) fingers of the nondominant hand (Fig. 15.5).

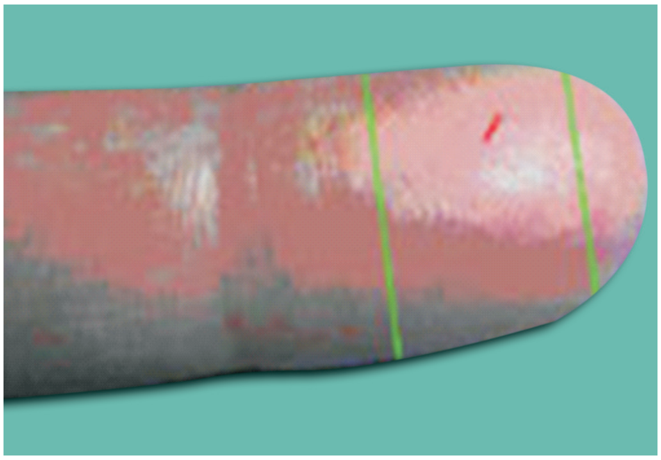


Fig. 15.5: Fingerstick procedure

- Do not use tip of the finger, or center of the finger. Avoid side of the finger, where there is less soft tissue, since vessels and nerves are located and the bone is closer to the surface. The 2nd (index) finger tends to have thicker, calloused skin. The fifth finger tends to have less soft tissue overlying the bone. Avoid puncturing a finger that is cold or cyanotic, swollen, scarred or covered with a rash.
- Using a sterile lancet, make a skin puncture, just off the center of the finger pad. The puncture should be made perpendicular to the ridge of the fingerprint, so that the drop of blood does not run down the ridges.
- Wipe away the first drop of blood, which tends to contain excess tissue fluid.
- Collect drops of blood into the collection device, by gently massaging the finger. Avoid excessive pressure that may squeeze tissue fluid into the drop of blood.
- Cap, rotate and invert the collection device to mix the collected blood.
- Patient should hold a small gauze pad over the puncture site for a couple of minutes, to stop bleeding.

- Dispose off contaminated materials/supplies in designated containers.
- Label all appropriate tubes at the patient's bedside.
- Deliver specimen promptly to the laboratory.

BLOOD COLLECTION ON BABIES

Recommended location for blood collection on a newborn baby or an infant is the heel. To draw blood from a baby:

1. Prewarm infant's heel (42° C for 3 to 5 minutes) to increase the flow of blood, for collection.
2. Wash your hands and put gloves on.
3. Clean the site, to be punctured, with an alcohol sponge.
4. Dry the cleaned area with a dry gauze pad.
5. Hold baby's foot firmly, to avoid sudden movement.
6. Using a sterile blood safety lancet, puncture the side of the heel in the appropriate region (Fig. 15.6).

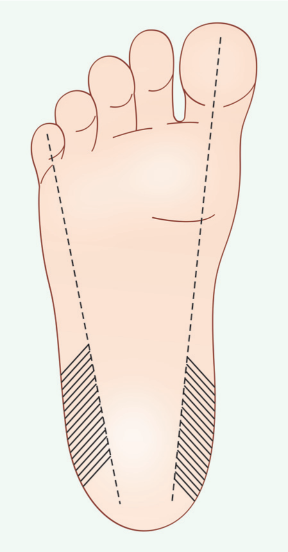


Fig. 15.6: Blood collection on babies

7. Make a cut across the heel print lines, so that a drop of blood can well up and not run down along the lines.
8. Wipe away the first drop of blood with a piece of clean, dry cotton gauze.
9. Since newborns do not, often, bleed immediately, use gentle pressure to produce a rounded drop of blood. Do not use excessive pressure because blood may become diluted with the tissue fluid.
10. Fill the required microtainer(s), as needed. When finished, elevate the heel, place a piece of clean, dry cotton on the puncture site and hold it in place until bleeding has stopped. Apply tape or Band-Aid to area, if needed.
11. Be sure to dispose off the lancet, in the appropriate container. Dispose off contaminated materials in appropriate waste receptacles.
12. Remove gloves and wash your hands.

Order of Filling Tubes after Withdrawal of Blood

As mentioned above, tubes in which blood is to be transported to the laboratory may contain a variety of additives (anticoagulants). Accordingly, it is important to know that which tube the individual laboratory requires for the desired test, as the type of reagents used and accordingly the anticoagulant, may vary between different laboratories.

The order in which various tubes should be filled, is determined by the risk of contamination and coagulation. The following sequence, for filling tubes with blood from a patient, is recommended to avoid contamination:

1. Vial for blood culture
2. Plain tubes (without any coagulant), for serum. Avoid serum as the first tube when electrolytes are to be measured.
3. Citrate containing tubes
4. Heparin containing tubes
5. EDTA containing tubes
6. Tubes containing additional anticoagulant, e.g. fluoride as glycolytic inhibitor.

Though, priority of the assay may affect order of filling of a particular tube but in general, specimen should be collected in the sequence, as shown in Table 15.1.

Table 15.1 Sequence of specimen collection

Type of analysis	Specimen type	Type of tube
Lipid profile	Serum	Plain tube
Glucose	Plasma	Glycolytic inhibitors, such as potassium oxalate and sodium fluoride
Glycated hemoglobin	Whole blood	EDTA.K_2

Additives must be thoroughly mixed. Erroneous test results may be obtained, if blood is not thoroughly mixed with the additive. It is important that before the subject leaves the collection site and that before the specimen rack is moved anywhere, all the tubes should be labeled with the individual's identifications.

Disposal of Needles and Other Materials

- Needle disposal box should be available for all personals, drawing blood samples.
- Needle should be released from the adapter directly to the needle disposal box.
- Needle should never be re-sheathed after use.
- The disposal box should not be allowed to become overfull, to avoid potential hazards.

LABELLING OF THE SAMPLE

A properly labeled sample is essential so that the results of the test match the patient. The key elements in labelling include:

- Patient's surname, first and middle name.
- Patient's OPD/CR number.
- Date and time.

Automated systems may include labels with bar codes (Fig. 15.7).

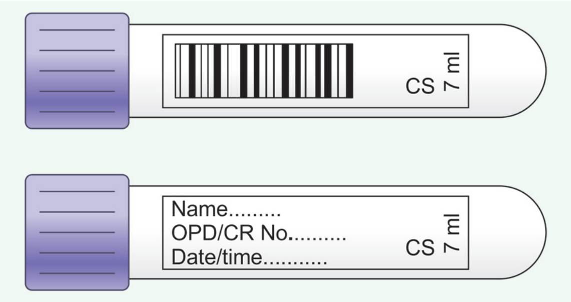


Fig. 15.7: Labelling of the sample

BLOOD SAMPLE HANDLING AND PROCESSING

Pre-centrifugation

First critical step in the lab testing process, after obtaining the sample, is the preparation of blood samples for analysis. Specimen integrity can be maintained by following some of the basic handling processes:

- Fill the tubes to the stated draw volume only and ensure proper blood to additive (anticoagulant) ratio.
- Allow the tubes to fill until the vacuum is exhausted and the blood flow has ceased.
- Vacutainer tubes should be stored between 4° and 25° C.
- Tubes should not be used beyond the designated expiration date.
- Mix all additive tubes by gentle inversion, 5 to 10 times, immediately after the blood has been added. This assists in the clotting process. This also assures homogenous mixing of the additives with the blood, in all types of additive tubes.
- Serum tubes should be left, to clot, for 30 minutes in a vertical position, prior to centrifugation. Short clotting time can result in fibrin formation, which may interfere with complete gel barrier formation.

During Centrifugation

It is recommended that serum should be physically separated from cells as soon as possible, with a maximum time limit of 2 hours, from the time of collection. Separation is time, temperature and force dependent (× *g*). For centrifugation, plastic tubes should be preferred over glass tubes.

For serum, blood samples are allowed to clot at about 25°C. Clotting time may be between 30 minutes and one hour. Clotted blood is generally centrifuged at 1500 × *g*, for 10 minutes. It should be centrifuged within one hour after blood collection.

For plasma, blood should be centrifuged as soon as possible and separated immediately (Fig. 15.8).

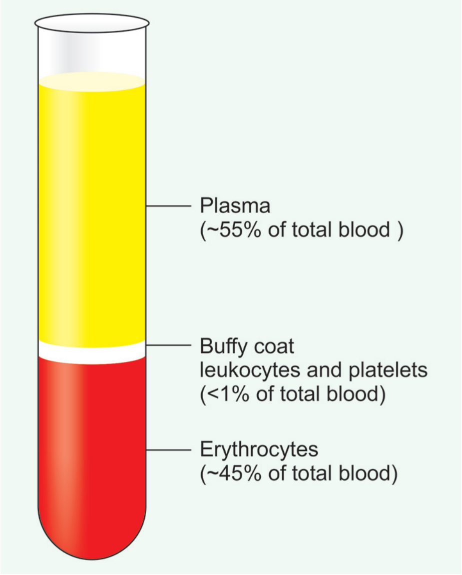


Fig. 15.8: Separation of plasma

Centrifuge the anticoagulant added blood (citrate, EDTA or heparinized blood) for at least 15 minutes, at 2000 to 3000 × *g*.

- In general, for a horizontal, swing-bucket centrifuge, the recommended spin time is 10 minutes. For a fixed-angle centrifuge, the recommended spin time is 15 minutes.
- Lid of the centrifuge machine should remain close, at all time, during the centrifugation process.
- Place the tubes in the centrifuge as a balanced load, noting the following that the:

  - Tube holders opposing each other, must be identical and should contain either the same type of cushion or none at all.
  - They must be loaded with equally weighted samples (tubes of the same size and equally filled in).
- If an odd number of samples are to be spun, fill a tube with water to match the weight of the unpaired sample and place it across this sample.
- Centrifuge must never be operated without a cover. Uncovered specimen tubes must not be centrifuged.
- Centrifuge must never be slowed down or stopped by grasping part(s) of the device with hand or by applying another object against the rotating equipment.
- Interference with an activated centrifuge by an impatient employee can result in bodily injury, in the form of direct trauma or aerosolization of the hazardous droplets.
- Be sure that the centrifuge is appropriately balanced before activating. If an abnormal noise, vibration or sound is noted, while the centrifuge is in operation, immediately stop the unit (turn off the switch) and check for a possible load imbalance.
- Clean the centrifuge daily with a disinfectant and paper towel. Broken tubes or liquid spills, if any, must be cleaned immediately.

Post-centrifugation

After centrifugation, tubes should be carefully inspected in order to recognize possible hemolysis, if any.

- Serum/plasma should be promptly separated from clot or cells and transferred to a clean tube.
- The white cell layer should not be transferred with the plasma.
- Amount of serum/plasma available for testing is, usually, less than 50% of the total volume of the blood withdrawn.
- After all the serum/plasma has been separated to the proper transfer/storage tubes, they should be carefully marked.
- If the sample is to be refrigerated for preservation, blood cells must be separated from serum or plasma.
- Noncentrifuged samples should not be stored at room temperature for longer time.
- Do not freeze whole blood sample before or after centrifugation.

HEMOLYTIC, ICTERIC OR LIPEMIC SAMPLE

Medical laboratory tests are affected by the endogenous as well as exogenous factors, which may be present in the sample matrix. Accordingly, the observation, such as hemolytic, icteric, opalescent, turbid or lipemic serum, should be documented for each sample, if some unusual color or turbidity is observed.

Hemolytic Sample

Hemolysis is defined as the release of intracellular components of erythrocytes and other blood cells, into the extracellular space of blood. After separation of the blood cells, hemolysis may be visible by the **red color of serum** or plasma.

- **Several cellular constituents**, with an intracellular concentration of 10 times higher than the extracellular concentration, e.g. potassium, lactate dehydrogenase, aspartate aminotransferase, etc. **will increase in plasma/serum after hemolysis**.
- Greater degree of hemolysis may also interfere with several other laboratory tests.

How to Prevent Hemolysis?

As mentioned above, hemolysis can interfere with many tests. Therefore, it should be avoided:

- Mix blood with anticoagulants gently (vigorous shaking can cause hemolysis) for 5–10 times.
- Avoid drawing blood from a hematoma, select another draw site.
- If using a needle and syringe, avoid drawing the plunger back too forcefully.
- Make sure that the venipuncture site is dry before the sample is withdrawn.
- Avoid a probing, traumatic venipuncture.
- Avoid prolong tourniquet application (not more than 2 minutes, less than 1 minute is optimal).
- Avoid massaging, squeezing or probing a site.
- Avoid excessive fist clenching.
- If blood flow into tube slows, adjust needle position to remain in the center of the lumen.

Icteric Sample

Bilirubin is present in plasma as free bilirubin as well as covalently bound to albumin. In addition, water soluble bilirubin conjugates (mono- and diglucuronides) also exist. When present at increased concentration in blood, conjugated bilirubin may also appear in urine. In patients with proteinuria, bilirubin bound to albumin may appear in urine.

- Bilirubin interferes in oxidase/peroxidase based test systems, i.e. depending upon its concentration, bilirubin reacts with H_2O_2, which is produced in the test system. This, in turn, gives lower values in

various enzymatic procedures, such as those which are used for the measurement of glucose, cholesterol, triglycerides, uric acid, etc.
- Bilirubin also competitively interferes with dyes binding to albumin.

Lipemic Sample

Lipemia is a turbidity of serum or plasma caused by the elevated lipoprotein concentrations and is visible to the eyes. For this purpose, a transparent sample container must be used to detect lipemia. Post-centrifugal coagulation of serum sample of heparinized patients can also be a cause of turbidity.

- Most often, lipemia is a result of increased triglycerides concentration in plasma/serum.
- This can be due to food intake, altered lipid metabolism or infusion of lipids.
- After intestinal absorption, triglycerides are present in plasma as chylomicrons for 6 to 12 hours. Lipemia in plasma or serum may be visually observed when triglycerides concentration is above 300 mg/dL.
- In whole blood, triglycerides concentration above 1000 mg/dL cause turbidity, which is visible by visual inspection.
- Centrifugation for 10 minutes at 1000 × *g* is effective when chylomicrons cause turbidity.

Transport/Storage of Samples

It is recommended that all the tests should be done on the same day. If all the tests are not possible on the same day, then:

- Total cholesterol, HDL cholesterol and triglyceride levels should be done on the day of sample collection.
- For tests, for which analysis is not possible on the day of sample collection, but within next 3 days, samples should be properly packed and cooled. They should not be frozen. If required so, they can also be transported in refrigerated packaging, at 4°C.
- If analysis is not possible within next three days, serum/plasma should be immediately frozen at –20°C. While transporting frozen samples, care should to be taken to avoid thawing.
- If it is anticipated that analysis cannot be done within 7 days, samples should be frozen immediately at –70°C.
- For the transport, samples should be properly marked and transfer lists should be kept in order to check for the possible disappearance of a sample.

PRACTICAL EXERCISES

1. Collection of blood sample and venipuncture
2. Sample labelling
3. Centrifugation
4. Preparation of collection tubes with different types of anticoagulants.

Important Questions

1. Describe the process of collection and handling of blood samples.
2. Differentiate between:
   i. Serum and plasma
   ii. Lipemic, icteric and hemolyzed sample
3. Write notes on:
   i. Venipuncture
   ii. Vacutainers
   iii. Blood collection on babies
   iv. Anticoagulants

Chapter

16

Infectious Biological Material

Infectious (biological) material is any material containing genetic information and is capable of reproducing itself or being reproduced in a biological system. Biological material may include **human tissue**, **biological fluids** (blood, sputum, urine, semen, vaginal secretions, cerebrospinal fluid, synovial fluid, pleural fluid, peritoneal fluid, amniotic fluid and faeces), **microorganisms** and **microbial toxins**.

Though no agent, that is a component of the normal or abnormal microbial flora of man, should be regarded as lacking totally in pathogenic potential and should be handled with appropriate techniques. Among the agents that have been identified in recent years as posing the greatest risk of infection to laboratory and ancillary personnel of diagnostic laboratories include **hepatitis B virus** (HBV), **hepatitis C virus** (HCV) and **virus of AIDS** (HIV), ***Mycobacterium tuberculosis*** and ***Shigella*** species.

Accidental spilling of liquid infectious culture is an obvious hazard, due to the generation of aerosols and/or small droplets. Besides, **biohazardous waste** (liquid or solid) generated through the handling of specimen may also contain infectious agents. Thus, all **biological material, including biohazardous waste**, should be handled with safety.

GENERAL CONSIDERATIONS FOR HANDLING OF INFECTIOUS SAMPLE

Individuals who work in a laboratory that handles infectious material are at risk of exposure to the substances they handle. The following general practices are required for all laboratories handling infectious substances:

1. Eating, drinking, smoking, storing of either food, personal belongings or utensils, applying cosmetics and inserting or removing contact lenses are not permitted in any laboratory. Contact lenses should be used only when other forms of the corrective measures are not suitably available.
2. Long hair should to be tied back or restrained, so that they do not come in contact with hands, specimens, containers or equipment.
3. Access to laboratory and support areas should be limited to authorized personnel only.
4. Doors to laboratories must not be left open (this does not apply to an open area within a laboratory).
5. Open wounds, cuts, scratches and grazes should be covered with waterproof dressings.
6. Laboratories must be kept clean and tidy. Storage of materials that are not pertinent to the work and cannot be easily decontaminated (e.g. journals, books, correspondence, etc.) should be minimized. Paperwork and report writing should be kept separate from such biohazardous materials work area.
7. Protective laboratory clothing, properly fastened, must be worn by all personnel. Suitable footwear with closed toes and heels must be worn in all laboratory areas.
8. Gloves must be worn for all procedures that might involve direct skin contact with biohazardous material and should be removed when leaving the laboratory. They should be decontaminated with other laboratory wastes before disposal.
9. Protective laboratory clothing must not be worn in nonlaboratory areas.
10. Laboratory clothing must not be stored in contact with street clothing. If a known or suspected exposure occurs, contaminated clothing must be decontaminated before laundering.
11. The use of needles, syringes and other sharp objects should be strictly limited. Caution should be used when handling needles and syringes to avoid auto-inoculation and generation of aerosols during use

and disposal. Needles should not be bent, sheared, recapped or removed from the syringe but should be promptly placed in a puncture-resistant sharp's container.

12. Hands must be washed after gloves have been removed, before leaving the laboratory and at any time after handling materials known or suspected to be contaminated.
13. Work surfaces must be cleaned and decontaminated with a suitable disinfectant at the end of the day and after any spill of potentially biohazardous material.
14. Contaminated materials and equipment leaving the laboratory for servicing or disposal must be appropriately decontaminated and lebeled or tagged-out, as such.
15. Efficacy monitoring of autoclaves, used for decontamination with biological indicators, must be done regularly.
16. All contaminated materials, solid or liquid, must be decontaminated before disposal or reuse. Such material must be contained in a way as to prevent release of the contaminated contents, during removal.
17. Disinfectants, effective against the agents in use, must be available at all times within the area where the biohazardous material is handled or stored.
18. Leak-proof containers must be used for the transport of infectious materials within laboratories.
19. Spills, accidents or exposures to infectious materials and losses of containment must be reported immediately.

COLLECTION, TRANSPORT AND RECEIPT OF INFECTIOUS MATERIAL

A large number of specimens are transported by various means between different hospitals and laboratories, every day. During transit, the containers or the packages, enclosing them, may be handled by many people. The containers may be involved in accidents and get damaged or broken. They may also be lost or opened by unauthorized persons. Although there are no reports of infections acquired this way, it follows that any infectious material being transported, could present a hazard to any person in contact with it. Therefore, collection, packaging, transport and unpacking should be strictly regulated.

Collection of Specimen

Containers

In most laboratories, disposable, screw-capped 25 mL bottles have replaced glass containers. Specimen containers must be sufficiently robust to withstand the stresses they are likely to meet and leakproof. Reusable containers (e.g. glass) must remain leakproof during their lifetime. They should be inspected before reassembly.

Blood

Traditionally blood is collected with hypodermic syringes and needles, and then expelled into the plastic containers. There are also problems with the stoppers of a lot of these containers, which result in external contamination. Needle stick injuries are, particularly, likely to occur in resheathing the needle. This practice should either be discouraged or a safer method of resheathing be used, perhaps with the aid of certain newly developed devices. Vacuum collection tubes minimize several of the hazards and problems of taking blood and disposing safely of syringes and needles. These containers are robust, leakproof and cannot be contaminated externally.

Faeces

Faeces are probably the most hazardous material, other than blood. These specimens are often collected by the patient, who is rarely instructed on the proper method of collection. Patients tend to fill the container with faeces and that fermentation may lead up to build-up of gas during transport. This may result in leakage or the cap coming off. Moreover, when such a container is opened, a violent broadcast may result. The other possibility is that outside of the container may be contaminated. Patients should be told to defecate on a pile of toilet paper in the lavatory pan and to remove a pea-sized portion with the spoon, to insert it carefully into the container and then to screw the cap on, tightly.

Other Specimens

Sputum specimens are probably less hazardous to handle because of the decreasing incidence of tuberculosis in developed countries. Apart from some viruses, organisms present in sputum are rarely infectious. Nevertheless, contamination of the outside of the container remains a problem. Risks from **urine** are minimal, although a small proportion of the patients may excrete *Salmonella typhi*, Leptospira, hepatitis or HF viruses. It is debatable, therefore, whether any expensive precautions are needed in the case of urine. External contamination is a problem and there is a strong case for collecting urine from females in sterile large jars before transferring it to a laboratory container. Other specimens, such as **pus** and **aspirated fluids**, must be collected by professional staff. It minimizes the risk of external contamination.

Labelling of the Specimens and Containers

Labels and stickers that are used should be self-adhesive for obvious reasons. Specimens labeled "**Danger of Infection**" should be placed in self-sealing plastic bags. Pins and staples should not be used as they may prick hands of any person handling the bag. Request form should be separate from the specimen.

Transport of Specimens

The leak-proof trays or boxes should be provided for the transport of specimens from wards to the laboratory and these should be able to withstand autoclaving or overnight exposure to disinfectant. Staff carrying the specimens should wear overalls and encouraged to hand wash, frequently. Taking of specimens into canteens and kitchens should be forbidden.

Receipt of Infectious Material in the Laboratory for Tests

Any specimen in a plastic bag, which carries a danger of infection label should not be removed from that bag. Leaking or broken specimen should not be touched, nor should with any other material in the same box or tray. Provision should be made for a member of the professional staff to deal with them. These specimens should not be allowed to stray to other parts of the room.

Harmful Effects of Infectious Biological Material

The main risk associated with working with an infectious biological material is the potential for infection. There are three main potential routes of infection.

Inhalation

Inhalation, e.g. breathing in a fine aerosol or vapour mist, may contain a viable organism.

Ingestion

Ingestion through poor hygiene practice, mouth pipetting or eating/drinking in the lab area.

Skin Penetration

This could be as a result of the injury with some contaminated sharp object, contact with mucous membrane of eyes/nose/mouth or entry via an uncovered wound.

Precautions to be taken while Handling Infectious Biological Material

Following precautions must be taken while using infectious biological material.

Use Protective Laboratory Coats and Gloves

The principal means of acquiring infections are through breaks in the skin caused by needle puncture, cuts or severe dermatitis by contamination of mucous membranes, and by inhalation. The risk of infection is decreased by preventing breaks in the body surfaces, preventing the formation of droplets that might contaminate surface breaks or mucous membranes, inserting barriers such as rubber gloves, goggles and masks between the infectious hazards and the potential site of entry, and preventing the generation of aerosols. Wearing of a lab coat, gloves, face shield, such as mask, safety glasses or goggles, protect against splashing infectious material onto the clothes, face and eyes.

Restrict use of Needles and Syringes

Restrict the use of needles and syringes only to those procedures for which there are no alternative. In such cases, use needles, syringes and other sharps carefully, to avoid self-inoculation. Dispose off sharps in leak-resitant and puncture-resistant containers only.

Pipetting of Specimen

A number of procedures carried out in the laboratory offer the potential for either direct or indirect exposure by the oral route. The procedure that offers the greatest potential for exposure by ingestion is mouth pipetting. Do not mouth pipette. Such exposures can be completely avoided through the use of mechanical pipetting devices.

Handling of Specimen

Special hazard warning labels be affixed to specimens that are known to be hazardous, e.g. specimens obtained from patient infected with hepatitis B virus (HBV), HCV or human immunodeficiency virus (HIV), or from patients in high-risk groups for these infections. Specimens for clinical laboratory examinations or culture should be handled in the same fashion as in patient care areas, with care being taken not to contaminate the outside of the container. Manipulate infectious fluid carefully, to avoid spills, production of aerosols and droplets.

Disposables

Disposables such as syringes, needles and knives, etc., should be placed in a leak-resistant and puncture-resistant container, for subsequent disposal. If persons are cut or punctured while dissecting or handling tissues or body fluids, the wound should be encouraged to bleed, flushed with abundant water and treated with an antiseptic, such as povidone-iodine.

Inactivation of the Infectious Material

Any spill of blood or body fluid should be cleaned immediately with a solution of household bleach, diluted 1:100 in tap water. Decontaminate work surfaces before and after use, and immediately after spill. Infectious material of the patient, particularly with HIV, HBV and HCV, should be readily inactivated by the use of formalin, hypochlorite or any iodine-based disinfectant.

Personal Hygiene

Do not eat, drink, chew gum, use tobacco, apply cosmetic or handle contact lenses in the laboratory. Do not store food for human consumption in laboratory refrigerators. Wash hands frequently after handling infectious materials, after removing latex/nitrile gloves and protective clothing, and always before leaving the laboratory. Keep hands away from mouth, nose, eyes, face and hair. Do not store personal items such as coats, boots, bags and books in the laboratory.

Health and Immune Status of the Laboratory Personnel

An important defense against infection with some viral agent is immunity induced by vaccination. Whenever a vaccine is available, its use should be considered for those at risk of exposure prior to their handling of the virus in question. All personnel working with infectious agents should have documented evidence of immunization with the vaccines for preventing infections.

Training and Supervision

Training and supervision are essential requirement for safe working with microorganisms. Training should cover the hazards of the work and the practical use of special procedures, techniques and equipment that are needed to minimize risks. Ancillary and cleaning staff should be instructed in safety procedures so that the hazards of the work area do not affect them. An appropriate level of supervision must be maintained.

Special Considerations for AIDS and Hepatitis Infectious Material

A universal approach should be adopted for the prevention of infections by blood and body fluid-borne organisms, such as hepatitis B virus (HBV) and HIV. The primary mode of infection is contact between the hands and the infected material, where the virus enters through cuts and abrasions.

Where standard precautions are used, there should be a set of standard operating procedures and only skilled staff should be employed. They should also receive HBV vaccine.

1. Precautions Against Contact

Protective clothing should be worn at all times and should be supplemented by plastic apron. Good quality disposable gloves should be worn. Cuts, scratches and abrasions on the hands should be covered. Masks, visors or goggles should be worn, if there is a possibility that the face may be splashed with blood or other material. Methods for the collection and transport of specimen should be reviewed to reduce, as far as possible, the number of operations that expose the operator and the possibility of leaking containers. Blood may be splashed when containers are opened, thus, suitable precautions should be taken, e.g. gripping the stopper or cap through a strip of paper wrapped around it, which will confine dispersal. Blood specimens should be centrifuged in sealed centrifuge buckets. If serum is separated by pouring, rim of the tube should be wiped with filter paper soaked in hypochlorite or glutaraldehyde. Care should also be taken in making and handling blood films. Sampling probes of automated apparatus should be wiped with tissues, held in gloved hands.

2. Precautions Against Injection

Accidental inoculation with hypodermic needle, other sharp instrument or broken glass may be reduced by replacing such hazardous equipment with inherently safer articles. Needles may be replaced by cannulas, glass Pasteur pipettes by the plastic disposable type and other glassware by rigid plastic or break-resistant glass. All re-usable glassware should be examined before it leaves the preparation room for chips and rough edges. Used sharp objects and broken glass should be discarded into rigid containers. Special precautions should be taken against needle stick accidents. Care is also essential when approaching the sharp sampling probes of some automated instruments.

3. Action in Case of Accidental Exposure

If skin is accidentally punctured or skin lesions are contaminated, immediate action is necessary. Bleeding should be encouraged under running water. The affected part should be washed immediately with soap and water. Mucous membrane and conjunctiva should be thoroughly irrigated with water.

4. Automated Equipment

Automated equipment should be of the closed system type, which is capable of being decontaminated by passing disinfectant through it. The probes should be shielded to avoid splashing. Effluent should be collected in closed bottles or discharged at least 25 cm into the waste plumbing system.

5. Inactivation of Specimen

The most commonly used method is heating the specimen to 56°C for 30 minutes, which is sufficient to inactivate HIV to undetectable levels. However, this is unlikely to have any effect on hepatitis B, which requires temperatures of up to 80–90ºC for inactivation. The cryostat should be well shielded, and the operator should wear face and eye protection. After use, machine should be brought to room temperature and disinfected.

6. Spillage, Decontamination and Disposal of Waste

If blood or other infected material is spilled, spillage should be immediately mopped up with gloved hands, and disinfected using either glutaraldehyde or hypochlorite (10000 ppm). HIV is effectively inactivated by alcohol mixture, hydrogen peroxide, hypochlorite and paraformaldehyde. Contaminated protective clothing should be autoclaved or bagged for hot wash laundering. Gloves should be discarded into contaminated waste receptacles for autoclaving or incineration. If street clothing is contaminated, it should be sponged with hot detergent solution, possibly with glutaraldehyde, depending on the nature of the material.

Important Questions

1. Describe general considerations for handling of an infectious sample.
2. Write notes on:
   - i. Collection, transport and receipt of infectious material
   - ii. Special considerations for handling of AIDS and hepatitis infected specimen.

Chapter

17

Disposal of Biological Waste

DISPOSAL OF BIOLOGICAL WASTE

It is the responsibility of the laboratory personnel to ensure safe and correct disposal of all the waste, produced in the course of their work. Improper and irresponsible disposal of chemical wastes down the drains, to the local authority refuse collection, or into the atmosphere is forbidden by the law. Due to new legislation, increasingly strict environmental controls and escalating cost of disposal, it is essential that appropriate disposal procedures should be strictly adhered. Different types of laboratory waste include:

- **Biological waste:** It includes biological material of human or microbial origin.
- **Biohazardous waste:** It includes infectious material, contaminated agar plates, live cultures, human cells and blood, and disposables, which have been in direct contact with the above materials.
- **Cytotoxic waste:** It includes a substance contaminated with any residue or preparations, which contains materials that are toxic to the cells, principally, for their action on cell reproduction. For example, staff preparing cytotoxic drugs for experimental purpose may be at risk of dermatitis, allergic reactions, cytogenic abnormalities, carcinomas, mutagenic effects to cells, etc.
- **Glassware:** All types of infected glassware, including those specifically designed for use in laboratory work.
- **Sharps:** Objects and devices, which have acute, rigid corners, edges, points or protuberances that are capable of cutting or penetrating the skin, e.g. hypodermic needles, glass, scalpel blades and lancets, are hazardous because of the potential to cause cuts and punctures. They may also be contaminated with toxic, infectious or radioactive material, which substantially increase the risk potential.
- **Winchester:** Brown glass container, most often large volume containers, which are used to store solvents, ammonia solution or concentrated acids.

General Considerations

- All generators of potentially hazardous waste must ensure segregation, accurate and complete labelling, and safe storage, transport, treatment and disposal.
- It must be ensured that the container is not leaking and that there is no spillage on the exterior of the container.
- Untrained staff and students should not handle hazardous waste.
- Personal protective equipment should be a consideration when handling a chemical waste.

Controlled Waste and Laboratory Waste Bins

All waste, suitable for the local authority refuse collection except recyclable paper and glass, is termed controlled waste. Items in this category, which includes dirty paper, plastic, rubber and wood, should generally be placed in the waste bins that should be available in each laboratory and may be collected by the cleaners.

Each laboratory must also have a container for certain items, which are not allowed to be put in the normal waste bins. In this special controlled waste container, one should put all broken laboratory glassware, any sharp objects of metal or glass, all fine powders (preferably inside a bottle or jar) and dirty sample tubes or other items, lightly contaminated with chemicals (but not any syringes or needles).

Laboratory controlled waste containers must be emptied regularly and should never be allowed to overflow. Under no circumstances, any item of glass, sharp metal or fine powder, should ever be put in a normal laboratory waste bin.

The tops (lids) must be removed from all bottles, which are put out for disposal and there should be no detectable smell of chemicals from any such bottle.

Wash Down Drains with Excess Water

The following material may be washed down the drain with excess of water:

- Concentrated and dilute acids and alkalis
- Harmless soluble inorganic salts (including all drying agents, such as $CaCl_2$, $MgSO_4$, etc.)
- Alcohol, containing salts
- Hypochlorite solutions from destroying cyanides, phosphines, etc.
- Fine (TLC grade) silica and alumina

Incineration (Solvent Waste Collection)

The following material requires incineration:

- All organic solvents, including water miscible ones
- Soluble organic waste, including most organic solids
- Paraffin and mineral oils (from oil baths and pumps)

Waste for Special Disposal

This is a troublesome and expensive method of disposal and the quantity of special waste must be kept to an absolute minimum. Only the following items should be disposed off in this way:

1. Schedule 1 poisons (but not cyanides) and other highly toxic chemicals
2. Materials contaminated with mercury
3. Carcinogenic solids, including asbestos.

Special waste must be collected in a separate labelled bottle or jar for disposal. On no account, must different types of waste be mixed.

The hoarding up of hazardous waste in laboratories is strictly forbidden.

Glass

For environmental reasons, recycling of glass is encouraged, but only certain items of waste glass from the laboratories are acceptable for recycling. Each laboratory should have a bin for recyclable glass. Only clean glass bottles, such as those in which chemicals are received, and broken or waste plate glass are allowed. All broken laboratory glassware items significantly contaminated by chemicals, sample tubes, droppers and glasswool, must be disposed off as controlled waste.

Biohazard/Sharps Disposal—Syringes and Needles

Sharps contaminated with biologically hazardous materials must be collected in special containers to be sent for incineration. Syringes and needles must not ever be put in a laboratory waste bin or controlled waste container.

BIOHAZARDOUS WASTE

Biohazardous waste, also called **infectious waste** or **biomedical waste**, is any waste containing infectious material or potentially infectious substance, such as blood. Of special concern are sharp wastes, such as needles, blades, glass pipettes and other wastes, that can cause injury during handling.

Biohazardous waste includes the following materials.

1. Human Blood and Blood Products

All human blood, blood products (such as serum, plasma and other blood components) in liquid or semi-liquid form, items contaminated with blood that, if compressed, would release blood in a liquid or semi-liquid form, or items caked with dried blood that are capable of being released during handling as well as other body fluids or tissues containing visible blood.

2. Human Body Fluids

Human body fluids in a liquid or semi-liquid state, including semen, vaginal secretions, cerebral spinal fluid, synovial fluid, pleural fluid, pericardial fluid, peritoneal fluid, amniotic fluid and saliva from dental procedures. Also, includes any other human body fluid, visibly contaminated with blood and all body fluids in situations where it is difficult or impossible to differentiate between body fluids.

3. Microbiological Waste

Laboratory waste containing or contaminated with concentrated forms of infectious agents. Such waste includes discarded specimen cultures, stocks of etiologic agents, discarded live and attenuated viruses, blood or body fluids known to contain infectious pathogens, wastes from the production of biologicals and serum, disposable culture dishes and devices used to transfer, inoculate and mix cultures.

4. Pathological Waste

All human tissues, organs and body parts, including waste biopsy material, tissues and anatomical parts from surgery, procedures, or autopsy, as well as any unfixed human tissue, except skin.

Table 17.1 Color codes for segregation of biomedical waste

Color	Waste	Treatment
Yellow	Human and animal anatomical waste/microbiology waste and soiled cotton/Dressings/Linen/Beddings, etc.	Incineration/Deep burial
Red	Tubings, cathers, IV sets	Autoclaving/Microwaving/Chemical treatment
Blue/white	Waste sharps (Needles, syringes, scalpels, blades, etc.)	Autoclaving/Microwaving/Chemical treatment and destruction/Shredding
Black	Discarded medicines/Cytotoxic drugs, incineration ash, chemical waste	Disposal in secured land fill

5. Gloves, Paper Towels, etc.

Gloves used to handle containers of blood or body fluids, **paper towels** or bench paper on which containers of blood or body fluids may have been placed but did not spill, or **any other material used to handle blood indirectly** but that did not come into direct contact with the blood.

Disinfecting and Disposing off Biohazardous Liquids

Biohazardous liquids and liquids that contain human blood should be disinfected in the lab and flushed down the drain. Do not attempt to disinfect solutions that contain a large amount of whole blood, to avoid clot formation in pipes.

To disinfect small amounts within the lab:

1. Add a disinfectant, such as Clorox (hypochlorite), until the solution of disinfectant and waste liquid is one-part disinfectant to five parts of waste liquid.
2. Cover all the objects in the liquid with the solution.
3. Make sure that there are no bubbles in the solution or on any submerged surfaces.
4. Let the solution stand for at least 30 minutes.
5. Pour the solution down the drain with cold water.

Segregation of Biomedical Waste

Segregate biological waste as noninfectious, infectious and sharps, and dispose them off in the recommended color coded disposal bags. Table 17.1 gives color codes for segregation of biomedical waste and the necessary treatment required.

Preparing Biohazardous Waste for Autoclaving or Disposal

Biohazardous (infectious) waste that cannot be treated in the lab, must be prepared and transported by lab personnel for treatment and disposal. Such waste also includes large amount of protein or clotted blood.

Important Question

1. Write notes on:
   i. Disposal of biological waste
   ii. Biohazardous waste
   iii. Color codes for biomedical waste
   iv. Disposal of biohazardous liquids

Chapter

18

Distilled Water

Distillation is a process of purification of water. It involves conversion of liquid to vapor and back to liquid, leaving the nonvolatile impurities in the vessel. Volatile impurities may be partially carried to the distillate. During the process, small water droplets may get entrapped in the vapor and may carry over and transport ions, like sodium, potassium, manganese, etc. to the distillate. Hence, water treated by distillation alone does not meet the specific conductivity requirements of grade-I water. Distillation involves a lot of heat expenditure.

Metal Distilled Water

The water distillation apparatus is used to prepare distilled water by using tap water. It is free from the interfering ions.

Components

- Distillation apparatus is made from stainless steel
- Steam condenser tube made from stainless steel
- Heating coil: It operates on 220 volts A/C, 50 cycles
- Inlet
- Overflow pipe
- Lid outlet

Principle

Tap water, in the still gets heated by heating coil. On boiling the vapors are formed which are collected in the condenser and are cooled by the circulated tap water. The condensed distilled water (DW) is free from the interfering ions and collected through the outlet (Fig. 18.1).

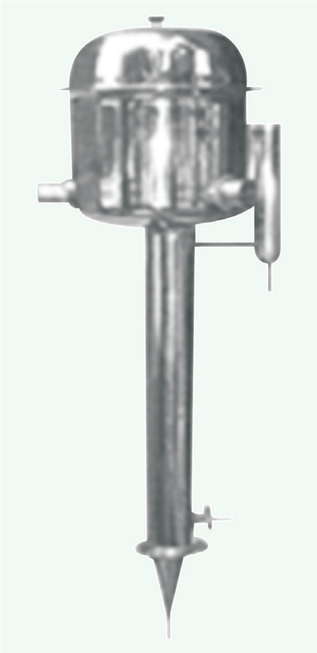


Fig. 18.1: Metal distillation apparatus

The yield of DW ranges from 4 to 8 L/hour. It may contain traces of sodium and chloride ions, and so is not suitable for electrolyte estimation.

Glass Distilled Water

Single Distillation Apparatus

This is produced by the process of distillation and has an electrical conductivity of not more than 11 µS/cm and total dissolved solids of less than 10 mg/L. Distillation involves boiling the water and then condensing the vapor into a clean container. The minerals are left in the distillation apparatus.

Apparatus Required

Two jars, glass tube having opening at both the ends, capillary tube, utensil big enough to hold the jar and burner (Fig. 18.2).

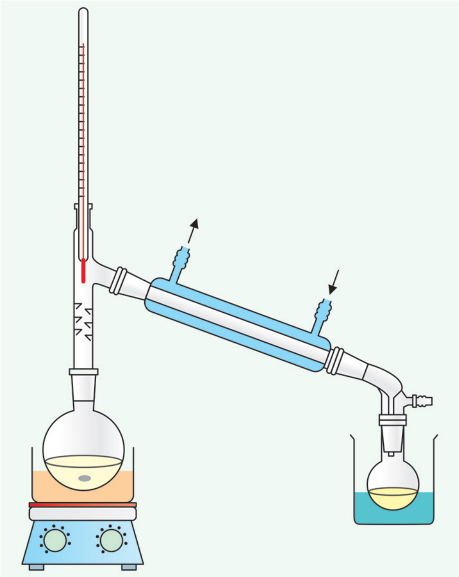


Fig. 18.2: Single glass distillation apparatus

Procedure

Fill one jar with tap water. Cover it with a lid, which should have an opening to insert the glass tube in it. Place the second jar inside a utensil and the jar should be covered with the lid having two holes in it. Now insert a glass tube through lids of both jars to connect them with each other. Insert a small capillary tube into empty hole of the lid of the second jar to make it act like a chimney for steam. Second jar should be covered with ice all around. Ice will act as a condenser for incoming steam.

Now boil the water of the first jar. The steam will rise and enter the second jar through the glass tube. It will get condensed into pure distilled water. Extra steam will escape through the capillary tube. This process of distillation is very slow.

Storage

Distilled water can absorb carbon dioxide from the atmosphere and can turn acidic over the time period, if not stored properly. Best is to store it in glass containers. In recent years, PVC containers are being used for storage but over a time period this water also absorbs carbon dioxide. High density polyethylene (HDPE) tanks have come up as an alternative in the recent years, for storage over a long period and at reasonable cost. These tanks are corrosive proof and are constructed with food grade approved resin to ensure safe storage of distilled water.

Single distilled water fails to meet the specifications for type 1 clinical laboratory grade water. The purity can be assessed and defined based on several factors.

Double Distillation

Double distilled water is prepared by slow boiling of the uncontaminated condensed water vapor from a prior slow boiling. The second distillation process occurs subsequent to the first distillation.

Triple Distillation

When double distilled water is subjected to reverse osmosis then water so obtained is said to be triple distilled water.

The double distilled water may also be treated with potassium permanganate. Some volatile organics co-distill with water and are destroyed by potassium permanganate which is a powerful oxidizer. It oxidizes the organic matter into water, CO_2 and nitrogen. This process also leads to formation of triple distilled water.

REVERSE OSMOSIS

It is a water purification technology that uses a semi-permeable membrane to remove ions, molecules and larger particles from the water. An applied pressure is used to overcome osmotic pressure and so the solute is retained on the pressurized side of the membrane and the pure solvent is allowed to pass to the other side.

In the normal osmosis process, the solvent naturally moves from an area of low solute concentration through a membrane, to an area of high solute concentration. The driving force for the movement of solvent is the reduction in the free energy of the system. Applying an external pressure to reverse the natural flow of the solvent, thus, is reverse osmosis.

Reverse osmosis (RO) is most commonly known for its use in drinking water purification, removing salt and other molecules from water.

DEIONIZED WATER

Water of the highest purity can be obtained by using deionizers. These are used in chemical, pharmaceutical and research laboratories.

The body of universal deionizers consists of a number of cylindrical vessels connected in series and charged with high grade purified cation and anion polyelectrolyte resins. It contains two separate flow circuits:

a. Water flow circuit for deionization

b. Regenerate flow circuit for the regeneration of the resins. While one circuit is on, other remains off.

The deionization of water does not require heating and the instrument is connected to the water tap and pure deionized water is obtained, and can be used for electrolyte determination.

STANDARD GRADES FOR PURE WATER FOR USE IN LABORATORY

The American Society for Testing and Materials defines 4 different standard grades for pure water to be used in laboratories. Various parameters of the four grades for pure water to be used in laboratories are described in Table 18.1.

- **Grade I** water is used for all special types of quantitative investigations like determination of electrolytes, blood gases, ELISA, RIA, reconstitution of lyophilized reagents, quality control sera and calibrators, and preparation of primary and secondary standards.
- **Grade II** can be used to perform routine qualitative tests.
- **Grade III** and **IV** can be used for glassware washing.

PRACTICAL EXERCISES

1. Preparation of metal distilled water
2. Preparation of glass distilled water

Table 18.1 Parameters of various grades for pure water for use in a laboratory (according to the American Chemical Society for Testing and Materials)

Parameter	Grade I	Grade II	Grade III	Grade IV
Conductivity mS/cm, 25°C	0.056	1	4	5
Resistivity, 25°C MΩ.cm	0.20	18.2	1	0.25
TOC (mg/L)	50	50	200	N/A
Total silica (mg/L)	3	3	500	N/A
Sodium (mg/L)	1	5	10	50
Chloride (mg/L)	1	5	10	50
pH	N/A	N/A	N/A	5–8
Special requirements for reagent grade water	Distillation or equal process followed by ion exchange and 0.2 mm filter	Distillation or equal process followed by ion exchange	0.45 μm filter	
Bacteria (CFU/mL)	10/1000	10/100	100/10	
Endotoxin (IU/mL)	<0.03	0.25	N/A	

Important Question

1. Write notes on:
   i. Metal distillation apparatus
   ii. Glass distillation apparatus
   iii. Reverse osmosis
   iv. Deionized water
   v. Standard grades for pure water for use in laboratory

Chapter 19

Reagents and Solutions

Quality analytical work can only be performed if all materials, including reagents, used in a clinical chemistry lab are suitable for the job, properly organized and well cared for.

Much of the success of an analysis depends on the reliability of the used standard and other reagent solutions. These should be prepared with great care and only by an experienced personal. When a standard or any other reagent solution is prepared, the bottle in which it is stored should be properly labeled. Distilled or deionized water should be used in making up all of these solutions.

PREPARATION OF REAGENTS

In spite of the era of automation in the clinical laboratory, where kits are being used, still a lab technician should know how to prepare various types of reagents for qualitative and quantitative tests. One of the most prominent sources of the errors made in a laboratory is the use of wrongly prepared or old reagents. Therefore, reagents have to be prepared very carefully and exactly, following the prescriptions. They have to be well labeled, including the date on which prepared.

Basic Requirements

- Solid and liquid chemicals are available as **analytical reagent grade chemicals** (AR grade) and **guaranteed reagent** (GR grade). Both of them are highly purified and should be used for the preparation of quantitative reagents. They should also be used in the preparation of normal and molar solutions, and primary standards.
- **General purpose chemicals** (GPR chemicals) can be used where purity within certain limits is required.
- **Laboratory reagents grade chemicals** (LR grade) can be used for preparation of qualitative reagents, like Benedict's reagent, Fouchet's reagent, etc.
- **Reagent grade water** should be used as a solvent for the preparation of most of the reagents and solutions in the laboratory.
- For the preparation of certain specific reagents required for electrolyte and hormonal studies, **glass distilled water**, which is prepared by glass distillation apparatus, should be used.
- Glacial acetic acid, isopropanol, chloroform and ethanol, etc. are commonly used as **solvents** in the lab.

Important Precautions to be Taken While Preparing Reagents

1. It is advisable to use **AR grade chemicals** in the laboratory.
2. When chemicals arrive in the laboratory, the containers need to be **labeled**. The label should be recorded with the date it was received, when it was first opened and, in some cases, the expiry date.
3. When **taking chemicals from a bottle** there are three basic rules to obey:
   - Use a clean spoon or spatula (do not use one which happens to lie around, unless it is cleaned).
   - Do not return unused solution to the bottle (to avoid contamination)
   - Close the bottles immediately and tightly after use to prevent evaporation
4. Use a watch glass to weigh hygroscopic chemicals like sodium hydroxide, trichloroacetic acid, etc.
5. After opening the sealed bottles of hygroscopic chemicals, it is convenient to dissolve the total contents of the bottle in distilled water to get stock solution which can further be diluted to get the desired solution as and when required.
6. Alkaline solutions can be stored in polyethylene bottles.

7. All the reagent bottles should be labeled appropriately along with the date of preparation and should be stored at an appropriate temperature.
8. Reagent solutions should generally not be kept for longer than six months after preparation (while some may only be used for few days and some need to be prepared freshly, each time they are used).

PREPARATION OF STANDARD SOLUTIONS

A standard solution is a solution containing a precisely known concentration of an element or substance, where a known weight of the solute is dissolved to make a specific volume. It is prepared using a standard substance referred to as a **primary reference material**.

Primary reference materials are highly purified chemicals, which are directly weighed to prepare a solution of known concentration. These chemicals, generally, are not hygroscopic and can be heated at 100°C. These solutions are **primary standards**. These reference materials can also be used to prepare or calibrate secondary reference materials. Both of these can be used for determination of analytes in the body fluids.

Primary standards are, generally, used in photometric and spectrophotometric determinations as reference solutions, to find out unknown concentrations in the specimen. For example, for blood glucose estimation, 100 mg/dL glucose standard is used. These are prepared in dilute acidic solutions to prevent the growth of microorganisms, e.g. glucose and urea standard is prepared in saturated benzoic acid, creatinine in 0.01 N HCl and cholesterol in glacial acetic acid, etc.

There are multiple ways to prepare a standard solution.

Molar Solution

A molar solution is formed by dissolving one mole of a substance in one liter of water.

Mole

Mole is a SI unit used to count number of particles in a reaction. Mole of oxygen has 6.0×10^{23} molecules of oxygen gas. Similarly, a mole of NaCl has 6.0×10^{23} molecules of NaCl, having equal number of sodium and chloride atoms. Mole of any substance has mass equal to the atomic or molecular mass depending on the composition. For example, one mole of calcium (an element) will weigh 40 grams while one mole of hydrogen gas (H_2) will weigh 2 grams. Similarly, one mole of a compound like NaCl will be equal to combined atomic mass of sodium (22.98 g) and chloride (35.45 g), i.e. 58.44 g or say 58.5 g (approximately). Since molar mass is described in grams, hence, it is also known as **gram molecular weight**.

Molarity = Moles of solute/liter of solution

If the molar solution is required in the form of M/2 or M/15, then divide the molecular weight of the chemical by 2 or 15 accordingly, and then dissolve the resultant amount in one liter of distilled water (DW).

Normal Solution

Normality of a solution is the number of gram equivalents dissolved per liter of solution. Gram equivalent can be calculated by dividing the molecular mass by valency. To make one normal solution of a substance, dissolve its one gram equivalent in water, to make final one liter solution.

Normality of a solution

$$= \frac{\text{Number of gram equivalents of solute}}{\text{Number of litres of solution}}$$

Since one g equivalent weight is equal to 1000 milliequivalent weight, so one normal solution = 1000 milliequivalent (mEq)/liter.

For an acid, it is the number of H^+ released and for base it is the number of OH^- released in the solution.

$$N = n \times M$$

For example, HCl releases one proton, so n will be = 1.

The normality and molarity are equal for HCl. Normality is used mainly for acids and bases and it is never less than its molarity.

Acid Solutions

To prepare common acid solutions, calculate the amount of the acid to make 1 L of acid solution.

For example, we have to prepare 1 N HCl. It is prepared by using commercially available concentrate HCl. Since the latter is available in the liquid form, the following method is used:

1. Specific gravity of HCl = 1.19
2. Percent by weight = 36
3. Approximate normality = 12.

$$\text{Actual acid content per mL} = \frac{1.19 \times 36}{100} = 0.42 \text{ g}$$

Since 0.42 g is present in 1 mL, then 36.6 g (equivalent weight of HCl) will be present in 87 mL of concentrate HCl. So, add 87 mL of concentrate HCl in about 900 mL of DW and make the final volume to one liter by using a volumetric flask. Its normality can be confirmed by titrating it against 1 N sodium hydroxide.

Preparation of 1 N HCl

1. Transfer 100 mL of DW with a measuring cylinder in a 250 mL beaker.
2. Add 10 mL of concentrated HCl and mix well.
3. Titrate it against a primary standard solution of 1 N NaOH.
4. Titration: For this purpose:
    - Pipette 2 mL of dilute HCl in a conical flask by using a volumetric pipette.
    - Add a drop of methyl orange.
    - Titrate against 1 N sodium carbonate till labeled of the reaction mixture changes from red to yellow.
    - Note the titration reading.
    - Take two more readings similarly.
    - Calculate the normality by the formula

    $N_1V_1 = N_2V_2$

    Suppose titration reading is 2.4 mL and the normality of the prepared solution is 1.2 and volume is 90 mL.

    Then by the formula:

    $N_1 \times V_1 = N_2 \times V_2$

    $1.2 \times 90 = 1 \times V_2$

    $V_2 = 108$ mL

    So, 90 mL of 1.2 N HCl has to be diluted to 108 mL, to get 1 N solution.

For preparing an acid solution:

- Always add acid to a large volume of water.
- The solution may then further be diluted with additional water, to make to one liter.
- Because mixing acid with water is an exothermic reaction, it is advised to use glassware, such as of borosilicate glass, which is capable of withstanding the change in temperature.

Base Solutions

- Stir the base into a large volume of water and then dilute the solution to make it to one liter.
- Use care when adding sodium hydroxide to water, since this is an exothermic reaction and generates considerable heat.
- It is advisable to use glassware, such as made of borosilicate glass and immerse the container in a bucket of ice to keep the heat down.
- Use solid sodium hydroxide and potassium hydroxide to prepare solutions of these bases.

Preparation of Primary Standard (1 N Sodium Carbonate)

1. Keep anhydrous sodium carbonate in the hot air oven at 110°C for one hour.
2. Then allow it to cool in a desiccator, to the room temperature (25° ± 5°C)
3. Weigh 5.3 g of it on analytical balance using a butter paper.
4. Transfer it to a beaker.
5. Dissolve it in distilled water, making the final volume to 100 mL by using a volumetric flask.

Preparation of 1 N Sodium Hydroxide

1. Weigh 4 g sodium hydroxide on an analytical balance.
2. Dissolve it in about 90 mL of DW in a beaker.
3. Titrate against 1 N hydrochloric acid solution.
4. Take at least 3 readings and find out the normality of sodium hydroxide.
5. If, normality is >1, then dilute it accordingly, and if <1, then add few more pellets.
6. Adjust normality by titrating against 1 N HCl
7. Store in a polyethylene bottle at room temperature.

Preparation of Common Reagents/Solutions

- **Hydrochloric acid solution from ampoule (0.1 M):** Use ampoules of 1 M HCl and dilute **10 mL** to **100 mL** with deionized water.
- **Hydrochloric acid solution from concentrate HCl (0.2 M):** In a fume-cupboard, add about 17 mL of concentrate hydrochloric acid, with stirring, to about 500 mL of water, and make up to 1 liter with water. Stopper, and mix thoroughly.
- **Sulfuric acid solution (1.5 M):** Add **85 mL** of concentrate sulfuric acid, slowly, to about **700 mL** of deionized water containing about 20 ice cubes. Stir the mixture and make up the volume with water to **1 liter** in a volumetric flask. Stopper the volumetric flask and invert several times.
- **Ammonia solution (3 M): In a fume-cupboard**, dilute **40 mL** of concentrated ammonia solution to **250 mL** with water.
- **Sodium hydroxide solution from ampoule (0.1 M):** Use ampoules of 1 M NaOH and dilute **10 mL** to **100 mL** with deionized water.
- **Sodium hydroxide solution** from pellets (0.1 M): Dissolve **4 g** of sodium hydroxide pellets in **50 mL** of cold water and make up to **1 liter** with water.

- **Sodium chloride solution (0.1 M):** Dissolve **5.85 g** of sodium chloride in water to make **1 liter** of solution.
- **Sodium chloride solution (saturated):** Add sodium chloride to water, with constant stirring, until no more will dissolve. A magnetic stirrer is very useful when doing this.
- **Silver nitrate solution (0.1 M):** Dissolve **4 g** of silver nitrate crystals in water and make up to **250 mL** with water. Store in a brown bottle. This solution is best, when freshly prepared and in very small quantities.
- **Sodium carbonate solution from anhydrous sodium carbonate (0.1 M):** Using a balance, weigh accurately **10.6 g** of pure anhydrous sodium carbonate on a watch glass. Transfer to a beaker containing about **200 mL** of deionized water. To ensure that all the sodium carbonate is transferred, use a wash bottle to rinse the watch glass with deionized water, and add the rinsings to the beaker. Stir the mixture with a stirring rod until the sodium carbonate has been fully dissolved. Using a wash bottle, wash off the solution on the stirring rod with deionized water into the beaker. Pour the solution through a clean funnel into a 1 liter volumetric flask. Using a wash bottle, rinse out the beaker several times with deionized water and add the rinsings to the solution in the flask. Rinse the funnel with deionized water, allowing the water to run into the flask. Carefully make up the volume to **1 liter** with deionised water. Stopper the flask and invert it several times to ensure a homogeneous solution.
- **Sodium carbonate solution from sodium carbonate decahydrate (0.5 M):** Dissolve **143 g** of hydrated sodium carbonate ($Na_2CO_3.10H_2O$) in water to make **1 liter** of solution.
- **Sodium hydrogen carbonate solution (0.5 M):** Dissolve **42 g** of sodium hydrogen carbonate (sodium bicarbonate) in water to make **1 liter** of solution.
- **Disodium hydrogen phosphate solution (0.03 M):** Dissolve **11.81 g** of disodium hydrogen phosphate in enough water to make **1 liter** of solution.
- **EDTA solution (0.1 M):** Dissolve **7.44 g** of the disodium salt of EDTA in enough water to make exactly **2 liters** of solution. Store the solution in a stoppered plastic bottle.

PRACTICAL EXERCISES

1. Preparation of 1 N solution of NaOH
2. Preparation of 1 N solution of HCl
3. Preparation of standard solution of glucose

Important Questions

1. Differentiate between a molar solution and a normal solution.
2. What precautions should be observed while preparing reagents?
3. Write notes on:
    i. Molar solution
    ii. Normal solution
    iii. Preparation of primary standard

Chapter

20

pH, Buffers and Indicators

pH

The pH refers to the value of H^+ ion concentration $[H^+]$ in an aqueous solution. Sorenson, in 1909, developed a scale by expressing the pH value in a logarithmic manner. He defined pH as the negative logarithm of $[H^+]$. Since this value is the **power of exponent**, the term "**p**" is used for this purpose. Accordingly,

$$pH = -\log_{10} [H^+]$$

To calculate pH of a solution:

- First, we calculate concentration of hydrogen ions, i.e. $[H^+]$, and then
- Find logarithm of it, to the base of 10.

For pure water, at 25ºC, the $[H^+]$ is 10^{-7}, hence

$$\begin{aligned} pH &= -\log 10^{-7}, \text{ or} \\ &= -(-7), \text{ or} \\ &= 7 \end{aligned}$$

Lower the pH, higher is H^+ concentration. On the other hand, higher is the pH, lower is the H^+ concentration. For every unit change in pH, there will be 10 times change in H^+ concentration.

Scale of pH Measurement

Sorenson's scale of pH extends from 0 to 14, taking the hydrogen ion concentration of 1 M to 10^{-14} M.

- The pH of a **neutral solution** is said to be **7.0**, whereas
- **Acidic solutions** have **pH <7.0**, and that
- The **basic solutions** have **pH >7.0**.

BUFFER SOLUTIONS

Buffer is a solution which **resists change in pH**; i.e. it minimizes a change in H^+ concentration **following the addition of an acid or a base**.

ACIDS

An acid is a substance whose dissociation in water **releases hydrogen ion** (H^+). Since the H^+ referred to as a **proton donor**. Addition of an acid to a solution thus, increases the concentration of free H^+ ions in the solution.

In aqueous solution, an acid, such as HA, reversibly dissociates into **H^+ ion** and a **conjugate base** (**A^-**). This dissociation of HA, thus, can be represented symbolically by the following equation:

$$HA \rightarrow H^+ + A^-$$

where A^- represents an anion (a conjugate base) that remains when H^+ (a cation) is released.

The tendency of an acid, to give up H^+ ion depends upon its strength.

Strong Acids and Weak Acids

A **strong acid**, such as HCl, completely dissociates in an aqueous solution, as follows:

$$HCl \rightarrow H^+ + Cl^-$$

On the other hand, a **weak acid** gives up H^+ ion less rapidly and does not dissociate completely.

$$H_2CO_3 \rightarrow H^+ + HCO_3^-$$

A solution of a weak acid, thus, contains both dissociated ions as well as undissociated molecule.

Most of the acids produced in the living systems are weak acids. Strength of an acid is denoted by its pK value which is low for a strong acid and vice versa.

BASES

A base **releases hydroxyl ion** (OH^-) in an aqueous solution and **decreases its H^+ ion concentration**, by accepting or binding with the free H^+ ions. A base, therefore, is referred to as a **proton acceptor**.

A **strong base** such as sodium hydroxide (NaOH) dissociates completely, as follows:

$$NaOH \rightarrow Na^+ + OH^-$$

The OH^- ions, so produced, accepts H^+ ions and result in the formation of water. This, in turn, lowers the concentration of H^+ ions in the solution.

$$OH^- + H^+ \rightleftharpoons H_2O$$

A **weak base**, such as bicarbonate (HCO_3^-), can also utilize H^+ ions, as follows:

$$HCO_3^- + H^+ \rightleftharpoons H_2CO_3$$

Amount of an acid or a base is expressed in terms of **equivalents**. One mole of HCl contains one equivalent of an acid and neutralizes one equivalent of the base. On the other hand, one mole of sulfuric acid (H_2SO_4) contains two equivalents of acid, since sulfuric acid can donate two H^+ ions and requires two moles of NaOH to neutralize it.

PREPARATION OF BUFFERS

A buffer is prepared by mixing **a weak acid and its salt with a strong base**, or **a weak base and its salt with a strong acid**, e.g. carbonic acid and sodium carbonate.

- As mentioned above, a weak acid such as HA, reversibly dissociates into **H^+ ion** and its **conjugate base** (**A^-**), i.e.:

$$\underset{\text{(Weak acid)}}{HA} \rightleftharpoons \underset{\text{(Proton)}}{H^+} + \underset{\text{(Salt or conjugate base)}}{A^-}$$

- The dissociation constant (Ka) of this acid is expressed as:

$$Ka = \frac{[H^+][A^+]}{[HA]}$$

- When an acid is added to such a solution, A^- neutralizes it and gets converted to HA.
- On the other hand, when a base is added to such a solution, HA neutralizes it and gets converted to A^-.
- A buffer has maximum buffering capacity at its pKa (+1), i.e. when acidic and basic forms are present in equal concentrations.

Buffer capacity is defined as the gram equivalent of acid or alkali required by one liter of a buffer, to undergo a change of pH by 1. Thus, a buffer requiring more acid or alkali is said to have more buffer capacity.

Preparation and Composition of Common Buffers

Various buffers are commonly required for use in all biochemical studies, especially in enzyme studies. Preparation and composition of various buffers, routinely used in a biochemistry laboratory are, as follows:

Acetate Buffer

It is mainly used in the pH range of 3.6–5.6. To prepare acetate buffer, we prepare a solution of acetic acid (A) and sodium acetate (B), and mix the two in different proportions, as shown in Table 20.1.

- **A**—0.2 M solution of acetic acid (11.55 mL/L)
- **B**—0.2 M solution of sodium acetate (16.4 g of $C_2H_3O_2$ Na, or 27.2 g of $C_2H_3O_2Na.3H_2O$/L)

Mix X mL of A + Y mL of B and dilute to a total of 100 mL.

Table 20.1 Preparation of acetate buffer

X	Y	pH	X	Y	pH
46.3	3.7	3.6	20.0	30.0	4.8
44.0	6.0	3.8	14.8	35.2	5.0
41.0	9.0	4.0	10.5	39.5	5.2
36.8	13.2	4.2	8.8	41.2	5.4
30.5	19.5	4.4	4.8	45.2	5.6
25.5	24.5	4.6			

Barbital Buffer (pH 6.8–9.2)

- **A**—0.2 M solution of sodium barbital (41.2 g/L)
- **B**—0.2 M HCl

50 mL of A + Y mL of B, dilute to a total of 200 mL (Table 20.2).

Table 20.2 Preparation of barbital buffer

Y	pH	Y	pH	Y	pH
1.5	9.2	12.7	8.2	32.5	7.4
2.5	9.0	17.5	8.0	39.0	7.2
4.0	8.8	22.5	7.8	43.0	7.0
6.0	8.6	27.5	7.6	45.0	6.8
9.0	8.4				

Note: Solutions more concentrated than 50 mM may crystallize on standing, especially in cold.

Borate Buffer (pH 8.1–9.0)

50 mL of 25 mM $Na_2B_4O_7.10H_2O$ (9.525 g/L) + Y mL of 0.1 M HCl, diluted to 100 mL (Table 20.3).

Table 20.2 Preparation of borate buffer

Y	pH	Y	pH	Y	pH
19.7	8.1	15.2	8.5	9.4	8.8
18.8	8.2	13.5	8.6	7.1	8.9
17.7	8.3	11.6	8.7	4.6	9.0
16.8	8.4				

Carbonate Buffer (pH 9.7–10.9)

50 mL of 50 mM $NaHCO_3$ + Y mL of 0.1 M NaOH, diluted to 100 mL (Table 20.4).

Table 20.4 Preparation of carbonate buffer

Y	pH	Y	pH	Y	pH
6.2	9.7	13.8	10.2	19.1	10.6
7.6	9.8	15.2	10.3	20.2	10.7
9.1	9.9	16.5	10.4	21.2	10.8
10.7	10.0	17.8	10.5	22.0	10.9
12.2	10.1				

Citrate Buffer (pH 3.0–6.2)

- **A**—0.1 M solution of citric acid (21.01 g/L)
- **B**—0.1 M solution of sodium citrate (29.41 g $C_6H_5O_7Na_3.2H_2O$/L; the use of the salt with $5H_2O$ is not recommended).

X mL of A + Y mL of B, diluted to a total of 100 mL (Table 20.5).

Table 20.5 Preparation of citrate buffer

X	Y	pH	X	Y	pH
46.5	3.5	3.0	23.0	27.0	4.8
43.7	6.3	3.2	20.5	29.5	5.0
40.0	10.0	3.4	18.0	32.0	5.2
37.0	13.0	3.6	16.0	34.0	5.4
35.0	15.0	3.8	13.7	36.3	5.6
33.0	17.0	4.0	11.8	38.2	5.8
31.5	18.5	4.2	9.5	40.5	6.0
28.0	22.0	4.4	7.2	42.8	6.2
25.5	24.5	4.6			

Glycine-HCl Buffer (pH 2.2–3.6)

- **A**—0.2 M solution of glycine (15.01 g/L)
- **B**—0.2 M HCl

50 mL of A + X mL of B, diluted to a total of 200 mL (Table 20.6).

Table 20.6 Preparation of glycine-HCl buffer

X	pH	X	pH	X	pH
5.0	3.6	11.4	3.0	32.4	2.4
6.4	3.4	16.8	2.8	44.0	2.2
8.2	3.2	24.2	2.6		

Glycine-NaOH Buffer (pH 8.6–10.6)

25 mL of 0.2 M glycine (15.015 g/L) + Y mL of 0.2 N NaOH, diluted to 100 mL (Table 20.7).

Table 20.7 Preparation of glycine–NaOH buffer

Y	pH	Y	pH	Y	pH
2.0	8.6	8.4	9.4	16.0	10.0
3.0	8.8	11.2	9.6	19.3	10.4
4.4	9.0	13.6	9.8	22.8	10.6
6.0	9.2				

Phosphate Buffer (Potassium Salt, pH 5.8–8.0)

50 mL of 0.1 M KH_2PO_4 (13.60 g/L) + Y mL of 0.1 M NaOH, diluted to 100 mL (Table 20.8).

Table 20.8 Preparation of phosphate buffer

Y	pH	Y	pH	Y	pH
3.6	5.8	16.4	6.6	39.1	7.4
4.6	5.9	19.3	6.7	40.9	7.5
5.6	6.0	22.4	6.8	42.4	7.6
6.8	6.1	25.9	6.9	43.5	7.7
8.1	6.2	29.1	7.0	44.5	7.8
9.7	6.3	32.1	7.1	45.3	7.9
11.6	6.4	34.7	7.2	46.1	8.0
13.9	6.5	37.0	7.3		

Sodium Carbonate-Bicarbonate Buffer (pH 9.2–10.8)

A—$Na_2CO_3.10H_2O$ (28.62 g/L)

B—$NaHCO_3$ (8.40 g/L)

X mL of A and Y mL of B (Table 20.9).

Table 20.9 Preparation of sodium carbonate–bicarbonate buffer

X	Y	pH	X 37°C	Y	pH 37°C
10	90	8.8	60	40	9.9
20	80	9.1	70	30	10.0
30	70	9.4	80	20	10.3
40	60	9.5	90	10	10.6
50	50	9.7			

Succinate Buffer (pH 3.8–6.0)

- **A**—Succinic acid (23.6 g/L)
- **B**—0.2 M NaOH

Mix 25 mL of solution A and Y mL of B, and dilute to 100 mL (Table 20.10).

Table 20.10 Preparation of succinate buffer

Y	pH	Y	pH	Y	pH
7.5	3.8	20.0	4.6	34.2	5.4
10.0	4.0	23.5	4.8	37.5	5.6
13.3	4.2	26.7	5.0	40.7	5.8
16.7	4.4	30.3	5.2	43.5	6.0

Tris–HCl Buffer (pH 7.2–9.0)

- **A**—0.2 M solution of tris (hydroxymethyl) aminomethane (24.2 g/L)
- **B**—0.2 M HCl

50 mL of A + Y mL of B, dilute to a total of 200 mL (Table 20.11).

Table 20.11 Preparation of tris–HCl buffer

Y	pH	Y	pH	Y	pH
5.0	9.0	21.9	8.2	38.4	7.6
8.1	8.8	26.8	8.0	41.4	7.4
12.2	8.6	32.5	7.8	44.2	7.2
16.5	8.4				

pH MEASUREMENT

The pH of solutions can be measured by the use of indicators and more accurately by pH meter.

The pH Indicators

An indicator is a substance which is used to determine the end point in a titration. In acid–base titrations, organic substances (weak acids or weak bases) are generally used as indicators. They change their color within a certain pH range, having different colors in the ionized and unionized forms. An indicator which is a weak acid is practically undissociated in acid solutions and gives the acid color. In presence of a dilute alkali, it will form salt, which will dissociate freely and display the alkaline color. At intermediate pH, the color will be a mixture of acid and alkali color.

Use of Indicators for pH Measurement

Several indicators exist for the measurement of acidity or alkalinity of a solution. These methods employ a change in the color, depending upon the hydrogen ion concentration (pH) of the solution. Each indicator has an effective range of pH over which it gives a detectable change in color. Usually, this range is one pH unit around its pK value. For example, bromothymol blue is blue at pH 7.6 or above and yellow at pH below 6.0. Thus, it is useful in the pH range of 6.0 to 7.6.

Universal pH Indicator

A universal indicator is the one which changes color differently at a wide range of pH variations. Most common composition of a universal indicator is methanol, propan-1-ol, sodium salt of phenolphthalein, sodium salt of methyl red, bromothymol blue (monosodium salt) and thymol blue.

This may be applied on strips of cellulose paper for dip test or used as aqueous solution, in a titration. The color changes when the universal indicator is used, is given in Table 20.12.

Table 20.12 Color changes during titration while using universal indicator

pH range	Nature of the indicator	Color of the solution
0–3	Very acidic	Red
3–6	Acidic	Orange/Yellow
7	Neutral	Green
8–11	Basic/base/alkali	Blue
11–14	Very basic/base/alkali	Purple

pH Papers

The pH paper consists mainly a variety of fungus, which is known as lichen. The biological name is **Rocchella tinctoria**. The dried lichen is powdered and is added with a neutral salt, which acts as an adhesive. This is coated on a cellulose fiber and cut into small strips (Fig. 20.1).

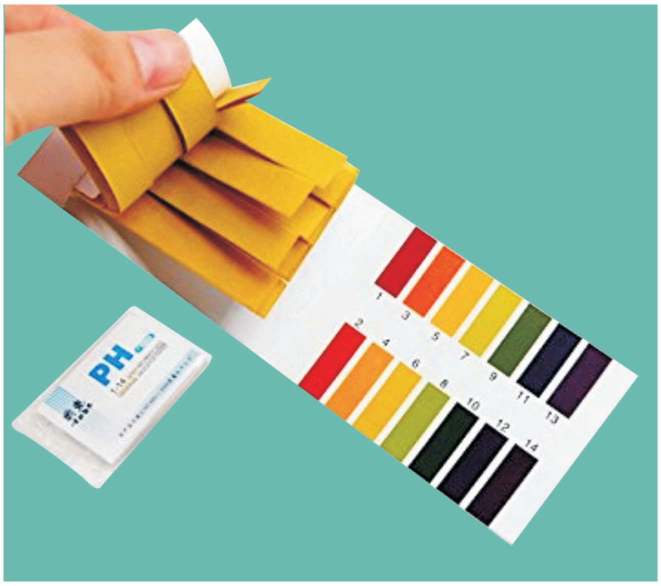


Fig. 20.1: Wide range pH paper

Types of pH Papers

There are two types of pH papers as given below.

Wide range

This is composed of a solution of several compounds that exhibit color changes over a pH value range from 0–14, to indicate the acidity or alkalinity of solutions. The pH unit graduations are of the order of 2 units.

Narrow range

The narrow range of pH papers offer high sensitivity and sharp color change for a solution within a specified pH range only. There are very narrow pH unit graduations of the order of 0.2–0.4 (Fig. 20.2).

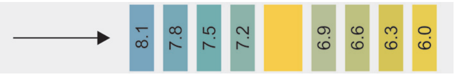


Fig. 20.2: Narrow range of pH papers

1. These strips are dipped in the solution and indicate approximate pH of the given solution.
2. It cannot be used in the titration since the sensitivity is not suitable for accurate pH reading.

Liquid pH Indicators

They are very easy to use and are mainly used for titrations. Add the number of drops of the indicator solution to the sample and match the reaction color that emerges with the corresponding color chart included with the indicator and read the pH value.

pH indicators are available in a wide range. The pH range and change in color of some common indicators are given in Table 20.13.

Table 20.13 pH range and change in color of some common liquid pH indicators

Indicator	pH range	Color change
Methyl orange	3.2–4.5	Pink to yellow
Methyl red	4.4–6.5	Red to yellow
Litmus	5.5–7.5	Red to blue
Phenol red	6.8–8.4	Yellow to red
Phenolphthalein	8.3–10.5	Colorless to pink

pH Measurement by use of Indicators

Pipette 5 mL of 1×10^{-3} M HCl stock solution into a beaker. Add 45 mL of distilled water to it. The H^+ concentration of the solution is now 1×10^{-4} (i.e. pH 4.0). Rinse the pipette with this solution and transfer 5 mL of it to 5 test tubes. Add 2 drops of each of the 5 different indicators to the solution in test tubes and note the change in color.

Take another solution of pH 5.0. Again, add indicators to the test tubes and note the colors. Repeat the procedure to get a solution of pH 6.0 to 10.0. Observe the color changes with the various indicators.

pH Measurement by pH Meter

A pH meter is an electronic method of pH determination, which gives an accurate determination of the pH of any solution. Several makes of pH meters are available in the market (Fig. 20.3).

Fig. 20.3: pH meter

Principle of pH Meter

In general, a pH meter consists of two electrodes, one glass electrode and the other calomel electrode. When two electrodes are kept in two different solutions, having differing H^+ concentrations, and the electrodes are connected to a potentiometer while the solutions by a salt bridge, a small potential difference is observed (emf). If one knows the standard emf produced by certain concentration of H^+, the emf of other solution can be measured by comparison.

Electromotive force is related to pH by the following equation:

$$pH = \frac{emf}{0.00019837\,T},$$

where *T* is the absolute temperature.

Hydrogen Electrode

The hydrogen electrode (platinum with platinum black) with hydrogen gas at one atmospheric pressure and immersed in a 1 N H^+ solution is assigned a potential of zero, under all conditions, and is used as the **standard reference electrode**.

Glass Electrode

It has been found that thin membranes of the glass are selectively permeable to H^+, and if a cell is set as follows:

a	b	c	d
Ag —— AgCl 0.1 M HCl		Unknown solution	$HgCl_2$–Hg saturated KCl
A	B	C	D

(H^+) and pH of an unknown solution can be determined, where:

- A represents a silver-silver chloride in half-cell containing 0.1 M HCl,
- C is the unknown solution to be tested,
- D is a standard calomel half-cell, and
- B is the glass membrane with a definite constant H^+ activity on one side (0.1 M HCl) and a variable H^+ activity on the other (unknown solution).

The potential across a glass membrane is a concentration potential due to difference in (H^+).

There are potentials at the boundaries a, c and d but these are constant for a given electrode and can be denoted as E′c. The value of this may be obtained by using a solution of known pH in C and measuring the potential E.

Now, the pH of any unknown solution can be obtained by the following formula:

$$\text{pH} = \frac{E - E'c}{0.00019837\,T}$$

where E is the emf seen in unknown solution, $E'c$ is the emf constant of the electrode, and T is the absolute temperature. In actual practice, an instrument is calibrated against a series of buffers of known pH.

PRACTICAL EXERCISES

1. Identification and use of different types of pH indicators.
2. Preparation of 0.1 M phosphate buffer and determination of its pH.

Important Questions

1. Define pH. What is the scale of pH measurement? Describe various processes for pH measurement.
2. Differentiate between acid and base.
3. Write notes on:
    i. Buffer
    ii. pH indicator
    iii. Universal pH indicator
    iv. pH papers
    v. pH meter

Chapter

21

Qualitative Analysis of Urine

Urine is an ultrafiltrate of plasma. It is formed in the kidney, a paired-organ, which is meant for **filtration**, **concentration** and **reabsorption** of various constituents. Each kidney has nearly one million basic filtration units called **nephrons**. A nephron consists of the **glomerulus** and the **tubule** (Fig. 21.1).

- The **glomerulus** is a network of arteriolar afferent and efferent capillaries and is surrounded by a double-walled epithelial sac called **Bowman's capsule**.
- Bowman's capsule leads to the **tubule**, which is divided into proximal convoluted tubule, loop of Henle and distal convoluted tubule.
  - **Loop of Henle** consists of the descending limb, a thin segment and the ascending limb.
  - **Proximal convoluted tubule** has its opening into the descending limb of the loop of Henle, which continues into the thin segment from where ascending limb arises.
  - Ascending limb of the loop of Henle continues into the **distal convoluted tubule**, which finally opens into the **collecting duct**.

Formation of Urine

- As blood passes through the **glomeruli**, noncolloidal substances (usually of low molecular weight) filter through capillaries, with the retention of protein (not normally found in the urine). About 150–200 L of the ultrafiltrate passes through the glomeruli in 24 hours. Reabsorption of water and solutes in the various regions of the **tubule** reduces its volumes to nearly 500–1800 mL per day.

  Total amount of the filtrate formed per minute by all the nephrons of both the kidneys is known as **glomerular filtration rate** (GFR). Under normal physiological conditions **GFR is 125 mL/min**.

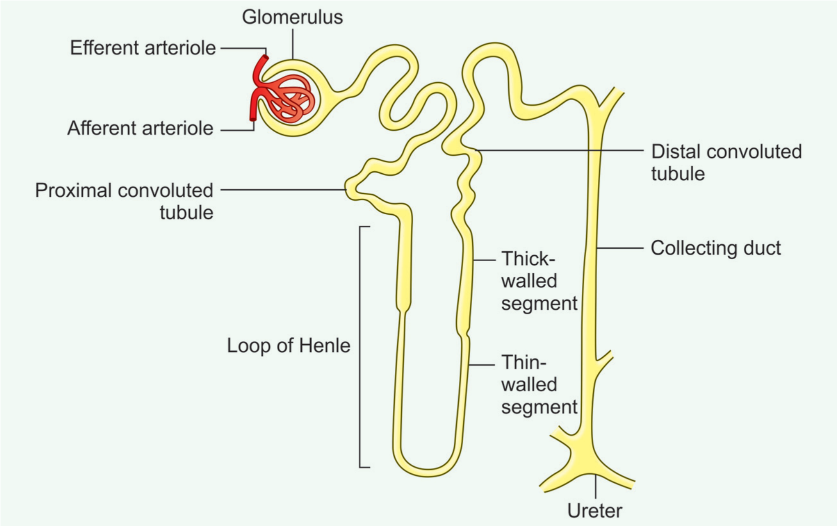

Glomerulus
Efferent arteriole
Afferent arteriole
Proximal convoluted tubule
Loop of Henle
Thick-walled segment
Thin-walled segment
Distal convoluted tubule
Collecting duct
Ureter

Fig. 21.1: Nephron

- Nearly 60–80% of the ultrafiltrate is absorbed in an obligatory fashion along with Na^+, Cl^-, HCO_3^-, Ca^{2+}, HPO_4^{2-} and other ions **in the proximal tubule**. Glucose and uric acid are also reabsorbed. When blood level of creatinine increases above normal, it is secreted in this region.
- Cl^- and some Na^+ are reabsorbed **in the loop of Henle**, but without water.
- Water reabsorption now occurs in the distal tubule and collecting duct, and is regulated by antidiuretic hormone (ADH or vasopressin).
- Secretion of organic ions, K^+ and H^+ occurs **in the distal tubule secretion**, as these are transported from blood, in the efferent arteriole, into the tubular fluid. Secretion of H^+ and reabsorption of Na^+ and HCO_3^- also occurs here.
- Hippuric acid and other organic waste products are also added here.

  Thus, urine carries:
  1. **Water** and **salts** in such amounts so as to maintain normal equilibrium between extracellular and intracellular fluid.
  2. **Acids** or **bases** to maintain a normal acid–base balance.
  3. **Waste products**.
  4. **Toxic** and **detoxified substances**.

COLLECTION AND HANDLING OF URINE SPECIMEN

Urine is an important tool for clinical diagnosis. The clinical information to be obtained from a urine specimen may be influenced by the collection method, timing and handling. A vast assortment of collection and transport containers for urine specimens are available. Determining which urine collection method and container should be used, depends upon the type of laboratory test ordered.

TYPES OF COLLECTION

Laboratory urine specimens are classified by the type of collection or collection procedures used to obtain the specimen.

First-morning Specimen

This is the specimen of choice for urinalysis and microscopic analysis, since the urine is generally more concentrated (due to length of time urine is allowed to remain in the bladder) and, therefore, contains relatively higher levels of cellular elements and analytes, such as protein, if present.

The first-morning specimen, also called an 8-hour specimen, is collected when the patient first wakes up in the morning, after emptying the bladder, before going to sleep. Alternatively, urine can also be collected over any eight-hour period for patients who have atypical work/sleep schedule.

Proper collection practices and accurate recording of the collection time are important criteria for the first-morning specimen. For this purpose, any urine which is voided from the bladder during the eight-hour collection period should be pooled and refrigerated, so that a true 8-hour specimen is obtained.

Random Specimen

This is the specimen most commonly sent to the laboratory for analysis, primarily because it is easiest to obtain and can be made readily available. This type of specimen is usually submitted for urinalysis and microscopic analysis, although it is not the specimen of choice for either of these tests.

It is due to the reason that random specimen can, sometime, give an inaccurate view of a patient's health, if the specimen is too diluted and analyte values are artificially lowered. Pediatric specimens, which routinely undergo chemistry and microscopic analysis, are generally of this type. As the name implies, a random specimen can be collected at any time. Although there are no specific guidelines for how the collection should be conducted, introduction of contaminants into the specimen should be avoided. This requires explicit instructions to patients so that they do not touch inside of the cup or cup lid.

Timed Collection of Urine Specimen

Among the most commonly performed tests requiring **24-hour urine specimen** include proteins, urea, creatinine, uric acid, VMA, 17-ketosteroids, etc. which are affected by diurnal variation. Sometimes, a timed specimen (usually 8-hour) may also be used for this purpose.

For the collection of urine sample over the fixed time, bladder is emptied prior to beginning the timed collection. Then, for the duration of the designated time period, all urine is collected and pooled into a collection container, with the final collection at the end of the period. Accurate timing is critical for calculations that are conducted to determine analyte concentrations and ratios. Interpretations based on faulty calculations can result in improper diagnosis and medical treatment.

Specimen Collection from Catheter

This procedure is conducted when a patient is bed-ridden or cannot urinate, independently. The healthcare provider inserts a Foley's catheter into bladder, through urethra, to collect the urine specimen (specimen may also be collected through an existing Foley's catheter). Specimen may be collected directly from a Foley's catheter into an evacuated tube or transferred from a syringe into a tube or cup.

Specimen from Suprapubic Aspiration

This method is used when a bed-ridden patient cannot be catheterized or a sterile specimen is required. The urine specimen is collected by needle aspiration through the abdominal wall into the bladder.

Pediatric Specimen

For infants and small children, a special urine collection bag is adhered to the skin surrounding the urethral area. Once the collection is complete, urine is poured into a collection cup or transferred directly into an evacuated tube with a transfer straw.

Urine collected from a diaper is not recommended for laboratory testing since contamination from the diaper material may affect test results.

URINE COLLECTION CONTAINERS

- Urine collection containers for 24-hour specimen may be of any shape and color but should be of 2–3 liters (L) capacity.
- They should be clean and free of particles or interfering substances.
- Urine collection container should have a secure lid and should be leak-proof. This will reduce specimen loss and healthcare worker's exposure to the specimen. It will also protect the specimen from contaminants. For this purpose, it is good to use containers that are made of break-resistant plastic, which is safer than glass.
- Specimen container should not be reused.
- Proper labelling should be applied to the collection container or tube.
- When a preservative is required, it should be added to the collection container before the urine collection begins. If there is more than one acceptable preservative for the analyte to be tested, the least hazardous one should be selected.

USE OF PRESERVATIVES

Some common 24-hour preservatives include hydrochloric acid, boric acid, acetic acid and toluene. They allow urine to be kept at room temperature, while still providing results comparable to those of refrigerated urine. Generally, length of preservation ranges from 24 to 72 hours. When the specimen is directly transferred from a collection cup into the preservative tube, it provides a stable environment for the specimen, until testing can be conducted, and reduces the risk of bacterial over growth or specimen decomposition. Nonadditive tubes (those not containing any chemical preservative) can be used for urinalysis but must be handled following strict timings and refrigeration guidelines.

SPECIMEN TRANSPORT

As with any type of laboratory specimen, there are certain criteria that need to be met for proper collection and transportation of urine specimen. This will ensure proper stability of the specimen and more accurate test results.

PHYSICAL EXAMINATION OF URINE

1. Volume

Volume of the urine varies from person to person and from time to time. Average volume of urine excreted by a normal individual, over a period of 24 hours, is **1000–1500 mL**. It is influenced by diet, particularly, ingestion of fluids. Strenuous physical exercise, hot weather and fever reduce urine output.

Certain pathological conditions also alter urine output. If 24 hours volume is increased, the condition is called **polyuria**, such as seen in diabetes and early renal failure. When urine output is decreased, it is termed as **oliguria** such as seen in acute renal failure, dehydration (diarrhea and vomiting), edema and urinary obstruction. In acute renal failure or shock, urine is usually not formed and the condition is referred to as **anuria**.

2. Color

Normal urine is usually **pale**, **straw-yellow colored** due to the presence of urochrome, uroerythrin and urobilin. Change in color may be seen in some pathological conditions, secondary to the intake of some drug or a particular type of food.

In fever, urine is usually **dark in color**. If there is **blood** in the urine (due to hemorrhage in the kidney or urinary tract), it becomes **red in color**. **In jaundice** urine may be **dark-yellow** or **green** due to the oxidation of bilirubin to biliverdin.

3. General Appearance and Odor

Freshly excreted urine is normally **clear and transparent** as all the substances are present in the soluble form. On standing, it becomes turbid due to the precipitation of phosphates. **Pathological urine may be turbid** due to the presence of fat globules or pus cells.

Odor of the urine is usually **aromatic** due to the presence of volatile acids. On standing urine may develop pungent odor due to formation of ammonia from urea because of its decomposition by bacteria. **In acidosis** and **diabetes mellitus**, urine has a peculiar **sweet smell**.

4. Specific Gravity

Specific gravity of urine normally varies between **1.010 and 1.030**. Volume has a great influence on specific gravity of urine. **Low values** (up to 1.003) are observed **after large water intake**, on diuretic therapy or excretion of large amount of ketone bodies. **High values** (up to 1.040) are seen in **dehydration** or due to the presence of large amount of glucose.

Specific gravity of urine is measured by means of hydrometer, known as **urinometer**. Mark on the urinometer in contact with surface of urine indicates specific gravity of the sample.

5. pH (Reaction)

Under normal circumstances urine is slightly acidic in reaction with a mean pH of **about 6.0** (ranging from **4.8–8.0**).

- The pH of urine varies widely depending upon the nature of the food taken:
  - **High protein diet** leads to **acidic** urine while **after a vegetarian diet**, particularly, after large intake of vegetables and fruits, urine is **alkaline**.
  - **Following meal** pH of the urine becomes **alkaline**, due to the production and secretion of HCl by the stomach.
- **Alkaline** urine is seen **in patients with systemic alkalosis** since body attempts to conserve H^+ ions and remove fixed bases.
- Strongly **acidic** urine is seen **in patients with systemic acidosis**, e.g. diabetes mellitus, due to the excretion of large amount of ketone bodies.
- Increase in pH **greater than 8.0** indicates **urinary tract infection** by an organism that splits urea to ammonia.

The pH of urine is routinely checked with the help of **pH paper**. Its reaction can be tested with the help of **litmus paper**.

CHEMICAL EXAMINATION OF URINE

1. Normal Constituents

Normal constituents of urine include a large number of solids, which include both organic and inorganic constituents. Normally 50–60 g of solids are excreted in urine over a period of 24 hours (Table 21.1).

- **Organic substances:** More than 50% of the total solids excreted are the organic substances. These include **urea**, **creatinine**, **uric acid** and **amino acids**. They are collectively referred to as nonprotein nitrogenous constituents (NPN).
- **Inorganic substances:** These include **chloride** (as sodium chloride), **ammonia**, **phosphates** and **sulfates**.

Table 21.1 Normal constituents of urine

Constituent	Daily excretion (g/24 hours)
Water	**1000–1500 mL/24 hours**
Total solids	**50–60**
Organic substances	**30–35**
Urea	25–30
Uric acid	0.5–1.0
Creatinine	1.0–2.0
Free amino acids (as amino acid N)	0.15–0.20
Inorganic substances	**20–25**
Chloride (as NaCl)	10–15
Total sulfates (as sulfur)	0.7–1.0
Total phosphates (as inorganic phosphorus)	0.8–1.1
Ammonia	0.5–0.7

INORGANIC CONSTITUENTS

Chloride

Chloride is a major inorganic constituent of normal urine. Its excretion is dependent upon chloride content of the food. On an average, daily output of chloride (expressed as sodium chloride) varies between 10 and 15 g. Fasting and excessive perspiration decreases its urinary excretion.

Pathologically, its excretion may be decreased in chronic nephritis, fever, burn, diarrhea and vomiting.

Test for Chloride

To demonstrate the presence of chloride in urine, take 5 mL of urine in a test tube. Add few drops of concentrate nitric acid and 2–3 drops of silver nitrate solution. A white precipitate, due to the formation of silver chloride is observed. It indicates the presence of chloride in urine.

Nitric acid is added to prevent precipitation of phosphates, urates and other organic radicals, which may be present in the urine.

Phosphates

Phosphates are present in urine in various forms which include:

- **Alkaline phosphates** such as sodium, potassium and ammonium phosphates, which are derived from the ingested inorganic phosphate.
- **Earthy phosphates** such as phosphates of calcium and magnesium. These are insoluble phosphates and get precipitated in alkaline urine.

Excretion of phosphorus is dependent upon diet but on an average **nearly 1.0 g** of phosphates (as inorganic phosphorus) are excreted **in 24 hours**.

Besides, urinary phosphorus is **increased in acidosis** and certain **bone diseases**, e.g. rickets and osteomalacia. Phosphorus excretion is **reduced in pregnancy**, renal and infectious diseases, and hyperparathyroidism.

Test for Phosphates

To demonstrate the presence of phosphate, take 3 mL of urine in a test tube and add few drops of concentrate nitric acid to it. Then, add 3 mL of 5% ammonium molybdate solution to it. A yellow precipitate of ammonium phosphomolybdate is seen, which indicates the presence of phosphates in urine.

Sulfates

Sulfates arise in urine from the oxidation of sulfur, which is obtained from sulfur-containing amino acids. Only a small amount of the ingested sulfur is excreted in urine.

Sulfates are present in the urine in various forms which include:

- **Inorganic sulfates** such as sodium, calcium and magnesium sulfates
- **Etheral sulfates** which are mostly the detoxification products
- **Neutral sulfates**, e.g. cystine, taurine, etc.

Under normal conditions, about 1.0 g of sulfates (as sulfur) are excreted daily.

Test for Sulfates

To demonstrate the presence of sulphate, take 5 mL of urine in a test tube and add 2–3 drops of concentrate hydrochloric acid. Add few drops of barium chloride solution. A white precipitate of barium sulfate indicates the presence of sulfates in urine.

Hydrochloric acid is added to prevent precipitation of phosphates and other salts of barium.

Ammonia

Tubular cells of the kidney form ammonia. Precursor for ammonia is the amide nitrogen of glutamine. Decrease in blood pH speeds-up ammonia production.

Normally, about 0.5–0.7 g of ammonia is excreted in 24 hours. Although very little of it is present in a fresh sample of urine, its level increases on standing, due to bacterial decomposition of urea.

Ammonia excretion is **decreased in a kidney disease** while its excretion is **increased in acidosis**, such as in diabetic ketoacidosis.

Test for Ammonia

To demonstrate the presence of ammonia, take 5 mL of urine in a test tube. Thereafter, cautiously, add dilute sodium hydroxide until the mixture is faint pink. Dip a glass rod in the solution of phenolphthalein. Boil the contents of the tube and hold the glass rod on the top of it. Phenolphthalein turns pink, due to vapors of ammonia.

ORGANIC CONSTITUENTS

Urea

Urea is the end product of protein metabolism. It is synthesized in the liver and removed by the kidneys.

Test for Urea

Presence of urea in urine is shown by alkaline hypobromite test where urea is decomposed, under the influence of sodium hypobromite to liberate CO_2 and N_2. CO_2 is absorbed by sodium hydroxide while the effervescence is observed due to evolution of nitrogen.

Take 5 mL of urine in a test tube and add 3–4 drops of the sodium hypobromite reagent. Effervescence is observed due to the evolution of N_2, which is liberated from urea.

Uric Acid

Uric acid is the end product of purine degradation. It is excreted in urine.

Test for Uric Acid

Presence of uric acid can be detected by using Benedict's uric acid reagent (alkaline phosphotungstic acid solution). Uric acid reduces this reagent and forms tungsten blue.

Take 2 mL of urine in a test tube. Make it alkaline by adding a pinch of sodium carbonate. Thereafter, add few drops of uric acid reagent and mix the contents. Deep blue color is observed.

Creatinine

Creatinine is the waste product excreted in urine.

Test for Creatinine

Creatinine is detected by alkaline picric acid solution. This is known as Jaffe reaction.

Take 5 mL urine in a test tube and add 1 mL of saturated picric acid solution to it. Make the mixture alkaline with the addition of 1 mL 10% sodium hydroxide solution. A reddish-orange color, due to the formation of creatinine picrate, is observed.

2. Abnormal Constituents

During the process of the filtration of plasma through glomeruli, large molecular weight substances such as proteins are retained while small molecular weight substances, which are useful for the body, are reabsorbed. Normally, their amounts are so low that they are not detectable by the routine laboratory tests. They are called **high threshold compounds** since they are vital for life and body cannot afford to lose them. However, under certain pathological conditions when either their metabolism is disturbed or tubular damage occurs, large quantities of these substances are excreted in the urine. Hence, these substances are called **abnormal constituents of urine**. These include sugar (glucose), ketone bodies, protein (albumin), blood, bile salts and bile pigments (Table 21.2).

Table 21.2 Abnormal constituents of urine

Constituent	Conditions in which excreted
Sugar (glucose)	Diabetes mellitus, renal glycosuria
Protein (albumin)	Nephrotic syndrome, glomerulonephritis
Bile salts/pigments	Different types of jaundice
Ketone bodies	Prolonged starvation, low-carbohydrate high-fat diet, diabetic ketoacidosis
Blood	Hematuria, hemoglobinuria

Sugar

Excretion of readily detectable amount of sugar in urine is known as mellituria or **glycosuria**. Excretion of glucose is also referred to as **glucosuria**.

When blood glucose concentration exceeds 180 mg/ 100 mL, such as in diabetes mellitus, reabsorptive capacity of the tubule is reduced and glucose starts appearing in urine. Besides glucose may also be found in urine in conditions such as hyperthyroidism, hyperpituitarism, hyperadrenalism and I cell disease of the pancreas, etc.

Renal Glucosuria

Glucosuria may also be observed in a proximal tubular disease that impairs reabsorptive mechanism. In this condition, glucose utilization as well as blood glucose level may be normal.

Test for Sugar

Presence of sugar in urine can be detected by Benedict test.

Take 5 mL of qualitative Benedict's reagent in a test tube and add 0.5 mL (8–10 drops) of the urine sample. Contents of the tube are boiled for 1–2 min and allowed to cool, thereafter. In the presence of a reducing sugar, red-colored precipitate of cuprous oxide is seen.

Depending upon quantity of sugar in the sample, contents of the tube may be seen as green, yellow, orange or red. The intensity of the precipitate can also be indicated by arbitrary number such as +, 2+, 3+ or 4+. Thus, this test not only indicates the presence of sugar in urine but also its approximate concentration and is a semiquantitative test (Table 21.3).

Table 21.3 Semiquantitative interpretation of Benedict's test

Color of the solution (with precipitate)	Arbitrary sign (unit)	Approx. concentration of sugar in urine (g/dL)
Green	+	Up to 0.5
Yellow	2+	About 1.0
Orange	3+	About 1.5
Red	4+	2.0 or above

Proteins

Normal urine contains only traces of protein which cannot be detected by routine procedure. When readily detectable amount of protein is seen in urine this condition is called **proteinuria**. Since it is mainly albumin that is detected abnormally, hence, proteinuria is a result of **albuminuria**.

Proteinuria or albuminuria may be physiological (benign albuminuria) or pathological (renal albuminuria).

Tests for Proteins

Presence of protein in the urine can be confirmed by various tests such as:

- **Sulphosalicylic acid test:** To 2 mL of urine, add few drops of 20% sulphosalicylic acid solution. White precipitate (turbidity) due to denaturation of albumin by sulphosalicylic acid indicates the presence of protein in urine.
- **Heat coagulation test:** Fill two-thirds of test tube with urine and adjust its pH to 4.6 (isoelectric pH of albumin). It may be done by adding a drop of chlorophenol red, followed by few drops of either dilute acetic acid (until red color is discharged) or dilute sodium carbonate (till pink color appears). Gently heat upper part of the fluid to boiling. Turbidity indicates presence of protein and phosphates.

  Add few drops of dilute acetic acid. Phosphates are dissolved. If turbidity persists, it is due to coagulation of albumin.
- **Nitric acid test:** Take 5 mL concentrate nitric acid in a test tube. Slowly add urine along the side of the tube so that it forms a separate layer above the acid. A white ring due to precipitation of proteins by nitric acid is observed. This is referred to as Heller's test.

Bile

Bile is produced in the liver. It is secreted through biliary tract and enters duodenum. Bile is alkaline in reaction and bitter in taste. Its color varies from yellowish-brown to dark green. Important constituents of bile include bile pigments, bile salts, lipids (particularly phospholipids and cholesterol), proteins like mucin, urea and certain inorganic salts, such as sodium chloride and bicarbonate.

Bile Pigments

These are the degradation products of hemoglobin. Bile pigments primarily include bilirubin and biliverdin.

Tests for Bile Pigments

Following tests are commonly performed to show to presence of bilirubin in urine:

- **Gmelin test:** Take 3 mL of fuming HNO_3 in a test tube and add 3 mL of urine (without disturbing the HNO_3 layer). Gently rotate the tube. At the junction of two fluids, multicolored rings are formed due to oxidation of bilirubin by HNO_3. Starting from the acid layer to the bile layer, the color is yellow, red, violet, blue and green.
- **Fauchet's test:** Take 5 mL urine in a test tube and acidify it by adding 1–2 drops of acetic acid. Add 5 mL of 10% barium chloride solution and 2–3 drops of saturated ammonium sulfate solution. Mix, allow to stand for 5 min, filter and collect the precipitate. Bilirubin is adsorbed on the precipitate of barium sulfate, giving it a yellow color. Unfold filter paper, allow extra water to drain (by laying on dry filter papers) and carefully add 1 drop of Fauchet's reagent on the precipitate. Blue-green color indicates the presence of bile pigments.

  Fauchet's reagent consists of trichloroacetic acid and ferric chloride. Ammonium sulfate and $BaCl_2$ react to form the precipitate of $BaSO_4$, which adsorbs bile pigments. They are released by trichloroacetic acid and react with $FeCl_3$, which oxidizes bilirubin (blue) to biliverdin and gives green color.

Bile Salts

Bile salts are sodium and potassium salts of glycocholic and taurocholic acids. Cholic acid has a basic steroid ring structure similar to cholesterol. In glycocholic acid, glycine is conjugated with cholic acid through a peptide linkage. Similarly, in taurocholic acid, taurine is conjugated with cholic acid. Bile salts are essential for the digestion of fat. Since fat is insoluble in water, it is not digested in the absence of bile salts. Bile salts emulsify fat by lowering surface tension of water and help to bring fat in intimate contact with the lipase.

Test for Bile Salts

Bile salts are the surface active agents. They form a layer on the surface of water and lower its surface tension. This principle is used to detect the presence of bile salts in urine and is referred to as **Hay's sulfur test**.

To demonstrate the presence of bile salts, fill the test tube with urine and sprinkle sulfur powder on the top of it. Sulfur sinks. When similar test is repeated with water, sulfur does not sink.

Due to surface tension (unbalanced force at the surface) sulfur powder floats on the surface. When bile salts are present, they lower surface tension and sulfur settles down.

Ketone Bodies

Ketone bodies (acetoacetate, β-hydroxybutyrate and acetone) are formed in considerable amount when fat is metabolized excessively. Their accumulation in blood is called **ketonemia** while their excretion in the urine is referred to as **ketonuria**. Ketonemia and ketonuria occur in a condition which is referred to as **ketosis**.

Ketosis may occur during prolonged fasting or carbohydrate deprivation. Pathologically, ketone bodies appear in urine, in diabetic ketoacidosis.

Test for Ketone Bodies

Ketone bodies in urine are detected by **Rothera's test** using sodium nitroprusside.

To demonstrate the presence of ketone bodies in urine, take 5 mL of urine in a test tube and saturate it with ammonium sulfate powder by shaking. Add 2–3 drops of 5% sodium nitroprusside sol ution to it. Mix and add few drops of liquid ammonia (ammonium hydroxide) from the side of the tube. Development of purple/violet color indicates the presence of ketone bodies.

Urobilinogen

Urobilinogen is an important constituent of the freshly voided normal urine. Following its oxidative degradation, urobilinogen disappears quickly. Increased amount of urobilinogen may be seen in urine of patients with hemolytic jaundice.

Test for Urobilinogen

Presence of urobilinogen is tested by Ehrlich's test.

To demonstrate the presence of urobilinogen in urine, add one mL of Ehrlich's reagent to the tube containing 10 mL of urine. A deep red color is seen. Color remains light red with the normal sample. Rapidity and intensity of the color gives an idea about the quantity of urobilinogen in urine.

Ehrlich's reagent is prepared by dissolving p-dimethylaminobenzaldehyde in hydrochloric acid.

Blood

Either whole red blood cells or hemoglobin may be present in urine.

- **Hematuria:** Presence of red blood cells in urine is referred to as hematuria. It is confirmed by the presence of red blood cells by microscopic examination of urine. RBCs may be seen in acute glomerulonephritis and trauma to urinary tract.
- **Hemoglobinuria:** It may be observed due to intravascular hemolysis. This, in turn, results in liberation of hemoglobin, which is filtered in the glomerulus and appears in the urine. Hemoglobinuria may occur under various pathological conditions such as mismatched transfusion of blood, hemolytic jaundice, typhoid or malaria.

Test for Blood

Presence of blood (hemoglobin) in urine may be shown by benzidine and O-toluidine tests.

To demonstrate the presence of blood in urine, take 3 mL of benzidine/O-toluidine reagent (saturated solution of benzidine or O-toluidine in glacial acetic acid) in clean dry test tube. Add 3 mL of hydrogen peroxide, mix and pour one-half of it to another tube. To one of the tubes, add few drops of water (to serve as control). To the other tube add few drops of urine. Tube containing urine shows deep-blue color.

The principle of the test is that hemoglobin acts as pseudoperoxidase and decomposes hydrogen peroxide. As a result of it, oxygen is liberated, which, in turn, oxidizes benzidine/O-toluidine and forms blue-colored complex. On exposure to air, blue color rapidly changes to brown within a few minutes.

Microalbuminuria

In patients with proteinuria, albumin excretion below 150 mg/dL may be found in early stages of diabetic nephropathy. This is referred to as microalbuminuria. It may be present in early stages when GFR may be normal and there is no evidence of glomerular lesions.

Specimen Collection and Storage

Early morning midstream sample or 24 hours urine should be collected, stored at 4°C and analyzed as early as possible.

Principle of the Test: Dye Binding Colorimetric Method

Pyrogallol red molybdate reagent complex reacts with protein to form a blue purple color, which can be measured at 600 nm. Optical density is proportional to the amount of albumin in urine.

Procedure

Take three test tubes and mark them as test (T), standard (S) and blank (B). Add different solutions, as shown in Table 21.4.

Table 21.4 Test for microalbuminuria

	T	S	B
Pyrogallol red molybdate reagent	2.5 mL	2.5 mL	2.5 mL
Urine	0.05 mL	—	—
Bovine serum albumin standard solution (50 mg/dL)	—	0.05 mL	—
Distilled water	—	—	0.05 mL

Mix well and incubate at room temperature for 15 min. Thereafter, measure the OD at 600 nm.

Calculation

$$\text{Urine protein (mg/dL)} = \frac{\text{OD (test)}}{\text{OD (standard)}} \times 50$$

Bence Jones Proteins

Light chain immunoglobulins, which are excreted in patients of multiple myeloma, are called Bence Jones proteins.

To show the presence of Bence Jones proteins, take 5 mL of the urine sample in a test tube. Heat it carefully by immersing in a beaker of warm water. Urine becomes turbid at 40°C and shows a precipitate up to 60°C. On increasing the temperature above 60°C precipitate disappears. It again reappears on cooling.

USE OF URISTIX

In clinical practice presence of an abnormal constituent can also be detected in the urine by the use of strips, called dipsticks. A dipstick is a thin strip of plastic with one or more cellulose pads fixed on it. Each pad is saturated with buffer and certain chemicals, which are present on the pad in the dry form. Hence, this technique is called **dry chemistry**.

Dipsticks are also referred to as **uristicks** (**uristixs**) since they are used for the analysis of urine.

Principle

Various chemicals, fixed on the pad, get activated when they soak water (from the urine sample). These reagents then react with the test component of the urine and change the color. Presence of an abnormal constituent in the urine can, thus, be detected and its approximate concentration can be found by comparing the color with the chart provided by the manufacturer.

Table 21.5 lists basic principle of each test with the use of dipsticks.

Table 21.5 Principle of and reagents used for various urine tests by uristixs

Test	Principle	Reagents used
pH	Double indicator	Methyl red and bromothymol blue
Protein	Protein error of indicators	Citrate buffer (pH 3.0), bromophenol blue and protein absorbent
Glucose	Double sequential enzyme reaction	Glucose oxidase–peroxidase and buffer
Ketone bodies	Sodium nitroprusside	Sodium nitroprusside and glycine buffer
Blood	Pseudoperoxidase activity of hemoglobin	O-toluidine, H_2O_2 and buffer
Bile pigments (bilirubin)	Diazo reaction	Diazo reagent
Urobilinogen	Ehrlich reaction	Buffered p-dimethylaminobenzaldehyde

Various types of dipsticks are commercially available. Some of these are specific for a particular test and have a single pad, whereas others have two or more pads, and are used for various test combinations.

Precautions

There are a number of precautions, which should be observed for the successful use of these dipsticks:

1. As reagents (most of the enzymes) have a fixed shelf life, dipsticks should not be used after the date of expiry.
2. Dipsticks should be properly stored in an area free from moisture, as moisture may inactivate the chemicals.
3. Test area of the strip (pad) should not be physically touched.
4. The test strip should be immersed in the sample only for a very short period (2 seconds) and should be tapped thereafter to remove the excess of water. Immersion for longer time may dissolve chemicals and wash the pad.
5. Allowable time, before color comparison, as per manufacturer, should be strictly complied with.

PRACTICAL EXERCISES

1. Detection of abnormal constituents of urine.
2. Estimation of microalbuminuria.

Important Question

1. Write notes on:
   - i. Nephron
   - ii. Formation of urine
   - iii. Physical examination of urine
   - iv. Normal constituents of urine
   - v. Abnormal constituents of urine
   - vi. Uristixs
   - vii. Microalbuminuria
   - viii. Bence Jones proteins

Chapter 22

Colorimetry

Colorimetry or colorimetry is the science (and technology) used to quantify and describe physically the human color perception. It is the measurement of the wavelength and the intensity of electromagnetic radiation in the visible region of the spectrum. It is used extensively, for identification and determination of concentration of substances that absorb light.

Two fundamental laws are applied. They are known as the **Lambert law**, which relates the amount of light absorbed and the distance it travels through an absorbing medium; and the **Beer law**, which relates light absorption and the concentration of the absorbing substance. The two laws may be combined and expressed by the equation:

$$\frac{\log I_0}{I} = kcd,$$

where I_0 = intensity of the incident beam of light, I = transmitted intensity, c = the concentration of absorbing substance, d = the distance through the absorbing solution, and k = a constant, dependent upon the absorbing substance, the wavelength of light used, and the units used to specify c and d. A simple application of this expression is found in comparing intensities of radiation transmitted through layers of different thicknesses of two solutions of the same absorbing substance, one with a known concentration, the other unknown. If the same incident intensity is used and if the thicknesses of the two solutions are adjusted so that the transmitted intensities are equal, then the concentration of the unknown (c_2) can be expressed by the ratio of the thicknesses of the two solutions, d_1/d_2, times the known concentration (c_1).

PHOTOELECTRIC COLORIMETER

In a photoelectric colorimeter a photoelectric cell, instead of the eye, is used to compare intensities. In photoelectric colorimetry, one of the factors may be variable while the other is held constant, i.e. thickness of solution and intensity of transmitted light. It is a device used to test concentration of a solution by measuring its absorbance at a specific wavelength of light. To use the colorimeter, different solutions must be made, including a control or reference of known concentration. The concentration of a sample then can be calculated from the intensity of the light passed through the sample.

A colorimeter is a light-sensitive device used for measuring the transmittance and absorbance of light passing through a liquid sample, i.e. it measures light transmission in units of absorbance or percent transmission. Colorimeters follow Beer law to provide the units of absorbance.

Beer law is a chemistry equation used to measure a sample's absorption by looking at the amount of light that enters and leaves the absorptivity, and the curvature of the sample's container. It states that the optical density of a solution is directly proportional to the concentration of the solute and optical path length through the solution. If I_0 is the intensity of radiation incident on a solution and I is the intensity of the transmitted light, then transmittance (T) is:

$$T = \frac{I}{I_0}$$

Accordingly, percentage transmittance = $T \times 100$.

The optical density (OD) of a solution is defined as the logarithm of reciprocal of transmittance, i.e.

$$\begin{aligned} \text{OD} &= \log 1/T, \text{ or} \\ &= 2 - \log T\ \% \end{aligned}$$

According to the Beer's law, $\text{OD} \infty C \times L$, where C = concentration of solution, L = path length of light through solution.

Therefore, OD = $\varepsilon \times C \times L$, where ε is a proportionally constant called extinction coefficient.

The concentration range over which a substance follows Beer's law, must be determined for each set of analytical conditions. Deviation from Beer's law may occur in the following conditions:

1. When concentrations are very high
2. When incident radiant energy is not monochromatic and radiant energy reaches the detectors without having passed through the sample, so, causing error in taking absorbance.

Working Principle

The colorimeter is based on Beer-Lambert law, according to which the absorption of light transmitted through the medium is directly proportional to the medium concentration. In a colorimeter, a beam of light, with a specific wavelength, is passed through a solution via a series of lenses, which navigate the colored light to the measuring device. This, in turn, analyses the color compared to an existing standard. A microprocessor then calculates the absorbance or percent transmittance. If the concentration of the solution is greater, more light will be absorbed, which can be identified by measuring the difference between the amount of light at its origin and that after passing the solution.

Absorption Process

Each different atomic and, hence, molecular structure of a chemical substance has unique resonant frequencies. When the frequency of the incident radiation on a substance coincides with this resonant frequency or energy level, its radiant energy is imparted to the substance. This phenomenon of energy transfer is referred to as **absorption**.

Liquids and other transparent material display color when they selectively absorb wavelength of radiant energy in the visible region. Although this effect can be observed by the eye only in the visible region, it exemplifies the absorption process in the neighboring UV and infrared regions. The amount of radiant energy absorbed by a sample, at a certain wavelength, depends upon the concentration of the sample.

When a monochromatic light passes through a solution that can absorb radiant energy of certain wavelength, the energy of transmitted radiant energy will be less than incident light. Some of the incident energy may be reflected by the cell (containing the solution) or absorbed by the cell as well as solvent. To eliminate these factors and to consider the absorption by the compound, a blank or reference solution is used.

Blank

It contains all the reagents except the substance to be analyzed. Thus, the blank tube helps to set zero. This overcomes the absorption of light by the solvent and the reagents.

Different types of blanks are used to identify the source of contamination in the sample, e.g. reagent blank, distilled water, etc.

Reagent Blanks

They are, particularly, essential with the pure standards and internal standards. For example, in the analysis of phosphate by the method of Fiske and Subbarow, the standard, usually employed, is monopotassium phosphate in trichloroacetic acid. Trichloroacetic acid used for making the protein-free filtrate of the serum sample, however, is not the same trichloroacetic acid present in the standard solution. Contamination of this reagent or the water used in the unknown in conjunction with trichloroacetic acid would be missed unless a blank were run.

Distilled Water

Distilled water can also be used to set zero but then the absorbance of a control solution, containing all the reagents, has to be subtracted from the test and standard values.

Where the absorption of a reagent blank is low, the reagent blank, standards and unknown should be read against distilled water, set at 100% transmission.

Standard

It represents known concentration of the substance to be estimated. Concentration of the standard range within the limits found in the specimen.

Components of a Colorimeter

1. **Light source:** In the visible range, usually, a tungsten lamp, while in the UV range, hydrogen, halogen or quartz lamp is used.
2. **Filters:** According to the electromagnetic theory of light, light travels in the form of waves described by three attributes, i.e. the wavelength, frequency and energy. The visible portion of the electromagnetic spectrum extends from 360 to 900 nm while the ultraviolet (UV) portion ranges from 200 to 380 nm. A filter isolates a rather broadband of wavelength at which the substances, to be estimated, absorb maximally. The isolated beam of light then passes through the sample placed in a cuvette of fixed path length, where a part of the incident energy is

absorbed so that the transmitted beam of light is reduced in intensity, in proportion to the concentration of the sample in solution. The transmitted beam of light then falls on a photocell or a phototube, which converts the light signal into an electrical signal that is finally read out as transmittance or optical density on a suitable meter.

It permits sufficiently narrow wavelength of light. Colored glass filters are used in simple instruments but in sophisticated ones, prisms or diffraction gratings are used. For each determination, a suitable wavelength is selected that can be attained by using the filters provided with the instrument. Use of the filter depends on the color of the resultant solution because absorbance is maximum when complementary filters are used (Table 22.1).

Table 22.1 Color of solution whose absorbance is to be read, the complementary color of the filter to be used and its wavelength range

Color of the solution	Color of the filter	Wavelength range
Bluish-green	Red	650–700 nm
Green-blue	Orange	600–650 nm
Blue	Yellow	575–600 nm
Violet	Yellow-green	555–575 nm
Purple	Green	505–555 nm
Red	Blue-green	495–505 nm
Orange	Green-blue	475–495 nm
Yellow	Blue	430–475 nm
Yellowish-blue	Violet	350–430 nm

3. **Cuvettes:** These are used to hold solutions whose absorbance is to be measured. They must be optically transparent, thoroughly clean and devoid of any scratch. Optical path in cuvette is always 1 cm. Glass cuvettes are used in visible range colorimetry while quartz or fused silica cuvettes are used in the UV range.

   In a manual colorimeter, the cuvettes are inserted and removed by hand. An automated colorimeter (as in an autoanalyzer) is fitted with a flowcell through which solution flows continuously.
4. **Photosensitive detector:** Photocell or a phototube is used to convert light energy into electrical energy.
5. **Measuring devices:** Any of the following read-out device can be used:
   - Galvanometer
   - Ammeter
   - Recorder
   - Digital readout

The output from a colorimeter may be displayed by an analogue or digital meter, and may be shown as transmittance (a linear scale from 0 to 100%) or as absorbance (a logarithmic scale from zero to infinity). The useful range of the absorbance scale is from 0 to 2 but it is desirable to keep within the range 0–1 because, above 1, the result becomes unreliable due to scattering of light. The output may be sent to a chart recorder, data logger or computer.

Most modern instruments are of direct reading type where the amplified detector signal operates a galvanometer (Fig. 22.1).

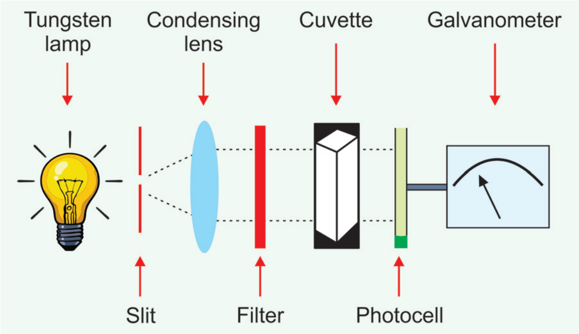


Fig. 22.1: Components of a colorimeter

SPECTROPHOTOMETER

A spectrophotometer is an instrument that measures the number of photons (the intensity of light) absorbed after it passes through a sample solution. With the spectrophotometer, the amount of a known chemical substance (concentration) can also be determined by measuring the intensity of light detected. A spectrophotometer works on the same principle as a colorimeter but it is more sensitive and sophisticated. The main **difference between colorimeter and spectrophotometer** is that **colorimeter** is a device which measures absorbance of specific color, whereas a **spectrometer** measures transmittance or reflectance as a function of wavelength.

A spectrophotometer, in general, consists of two devices; a spectrometer and a photometer.

Spectrometer

It is a device that produces, typically disperses and measures light. It produces a desired range of wavelength of light. First a lens transmits a straight beam of light (photons) that passes through a monochromator (prism) to split it into several component wavelengths (spectrum). Then a wavelength selector (slit) transmits the light of only the desired wavelength.

Photometer

A photometer indicates a photoelectric detector, which measures intensity of light. After a desired range of wavelength of light passes through the solution of a sample in the cuvette, photometer detects the amount of photons that is absorbed and then sends a signal to the galvanometer or some digital display.

Depending on the range of wavelength of light source, it can be classified into two types.

UV-Visible Spectrophotometer

It uses light in the ultraviolet (185–400 nm) and visible range (400–700 nm) of electromagnetic radiation spectrum.

IR Spectrophotometer

It uses light over the infrared range (700–1500 nm) of electromagnetic radiation spectrum.

Most common spectrophotometers are used in the UV and visible regions of the spectrum (400–700 nm). They take readings in the range of every 5–20 nm along the visible region and produce a spectral reflectance curve. Further, they operate best at the range of 0.2–0.8 OD.

- A brief explanation of the procedure of spectrophotometry includes comparing the absorbency of a blank sample that does not contain a colored compound, to the sample that contains a colored compound. If the compound is more concentrated, more light will be absorbed by the sample. Accordingly, within the small range, Beer-Lambert law holds and absorbance between the samples vary with concentration, linearly.
- Spectrophotometry is an important technique used in many biochemical experiments that involve DNA, RNA and protein isolation, enzyme kinetics, and biochemical analysis.
- Samples are usually prepared in cuvettes, which may be of glass or plastic (for visible spectrum region) or quartz (far UV spectrum region).
- The spectrophotometer is set-up according to an in-house standard operating procedure, calibrated by zeroing against air (i.e. no sample cuvette in the cuvette holder) and setting 100% T against a blank solution (Fig. 22.2).

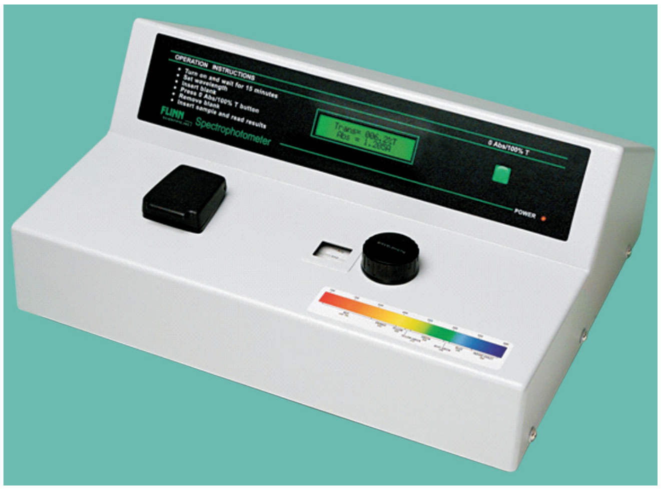


Fig. 22.2: Spectrophotometer

Precautions to be Taken while Using a Spectrophotometer

- Turn the power indicator light on, allowing the lamps and electronics to warm for 20 minutes. Adjust the selection switch to "T".
- Adjust the correct wavelength of the test required.
- Carry out the set-up procedure in the correct order.
- Wipe out the fingerprints and split sample of the outside of the cuvette before measuring %T. Thereafter, perform calibration checks after set up.
- Close door of the cuvette compartment, before reading the result.
- Open the sample compartment lid (light automatically close the door), transfer distilled water into the cuvette and adjust the "0% T" knob, so that the number appear as "00.0".
- Cover the sample compartment lid and place the reference solution cuvette in the light path, adjust the transmission of "100% T" knob, so that the numbers appear as "100.0 T".
- Record data, according to the formula, to calculate results.
- Clean up any spills, from inside the cuvette compartment.
- After determination of the sample compartment, close the lid.
- Clean the cuvettes, close the switch and the power supply.
- A particular instrument cuvette may not support the other instrument.

SEMIAUTOANALYZERS

These analyzers are so called because the initial stages of a specimen analysis like pipetting of reagent, pipetting of specimen, mixing and incubating the reaction mixture are performed by the laboratory technician.

Functions of Semiautoanalyzers

- Reading end point, rate of reaction in mono- and bichromatic modes.
- Displaying test results, printing and saving these results and graphs.

Features

Allows routinely performed and special biochemical tests like **hormonal assay, electrolytes, therapeutic drugs and drug-enzyme investigations** to program directly through keyboard (Fig. 22.3).

- Economical methodology in terms of sample volume (200–500 microliters)
- Lists all the standard operation procedures.
- Plots absorbance versus wavelength for selected range.
- Performs statistical analysis like mean, SD, CV, etc., and can plot Levey-Jennings charts for quality management.
- The semiautoanalyzers are very suitable for medium level laboratories. They are cheap and compact.
- Enzyme determinations by kinetic methods can be performed accurately over a period of 1–3 minutes.

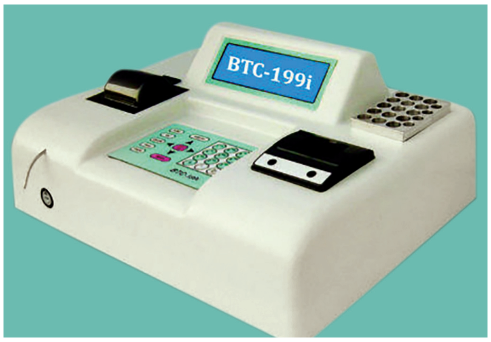


Fig. 22.3: Semiautoanalyzer

Types of Semiautomated Analyzers

There are three different versions of semiautoanalyzers. These include:

1. Semiautoanalyzer with built in dry block at 37°C with about 12 cuvette positions and one for reading. Cuvettes are handled by manual operations.
2. Semiautoanalyzers with built in fixed metal thermo-cuvette (with quartz windows), operated by automatic aspiration and washed by peristaltic pump.
3. Analyzers with following triple cuvette system
   - 33 ml flow cell cuvette
   - Manual square cuvette
   - Manual round tube for coagulation and ELISA test.

Components of Semiautoanalyzer

Here the samples and reagents are mixed and read manually.

- **Sample suction pump:** It sucks the sample and reagent mixture by a tube at the end of reaction and collects it in a cuvette.
- **Cuvette/flow cell:** Made up of glass or quartz. Used to contain the sample reagent mixture in order to take an OD.
- **Software:** Includes main interface, parametric setup, patient performance panel, testing panel, etc.
- **Filters:** Ultraviolet filters for taking reading beyond visible range.
- **Lamp:** Halogen lamp is commonly used.
- **Data management software:** Used to calculate and store data.
- **Storage device:** Used to store data like calibration, etc.
- **Keyboard and mouse:** Input device
- **Monitor:** It is a screen which displays results and all other necessary softwares.
- **Inlet water supply:** It is connected with a pure water source appropriate for the analyzer.
- **Water outlet:** It is connected with a drainage system.

STANDARD CALIBRATION CURVE

A calibration curve is a general method for determining the concentration of a substance in an unknown sample by comparing the unknown to a set of standard samples of known concentration (standard solution). For this purpose, several dilutions of a solution of known concentration, i.e. a standard solution, are prepared and analyzed by using a colorimeter. Their readings (concentrations) are then plotted on a graph, against absorbance, thereby preparing a calibration curve. Results of the unknown samples are then compared to that of the known sample from the curve, to measure the concentration. For most of the analysis, a plot of absorbance (instrument response) versus concentration shows a linear relationship.

Table 22.2 Preparation of the test tubes for preparing the standard curve for protein

	Protein standard (mL)	Distil water (mL)	Biuret reagent (mL)
Blank	—	2.5	3.5
T_1	0.5	2.0	3.5
T_2	1.0	1.5	3.5
T_3	1.5	1.0	3.5
T_4	2.0	0.5	3.5
T_5	2.5	—	3.5

Preparation of a Standard Calibration Curve (for Protein)

Take six test tubes, and label them as blank and T_1–T_5. Add protein standard, distil water and biuret reagent as shown in Table 22.2.

Wait for 10 min and measure the OD of each solution at wavelength 540 nm after setting zero with blank. Plot a graph taking concentration on X axis and OD on Y axis.

Linear graph will verify the Lambert-Beer law.

Important Questions

1. What is a colorimeter? Describe various components of a colorimeter with diagram.
2. Write notes on:
   i. Spectrophotometer
   ii. Standard calibration curve

Chapter 23

Routine Clinical Chemistry Investigations of Blood

BLOOD GLUCOSE

There are several photometric and oxidation-reduction methods, besides the enzymatic method, to measure blood glucose concentration. Some of these, such as Folin-Wu method, is relatively nonspecific and results in falsely increased values.

OXIDATION-REDUCTION METHODS

Many procedures for measuring glucose are based on its property of acting as a reducing agent in a hot alkaline solution. The principle of these methods is that in a hot alkaline solution aldehyde group of glucose readily reduces cupric ions (Cu^{2+}) to cuprous ions (Cu^{+}), which, in turn, form cuprous oxide (Cu_2O). Standard solution is also analyzed in the same manner, as protein-free filtrate, due to the reason that this reaction is dependent on the alkalinity, time and temperature of heating, and concentration of reagents. Thereafter, phosphomolybdic or arsenomolybdic acid is added, which, in turn, is reduced by the cuprous ions and forms molybdenum blue (this has lower oxidation state of molybdenum and is blue in color). The intensity of the color is measured colorimetrically.

Most commonly used oxidation-reduction methods include Folin-Wu method and Somogyi-Nelson method.

Folin-Wu Method

It is very old method and is not being used in most of the laboratories.

Principle

Firstly, proteins are precipitated with tungstic acid and the protein-free filtrate is used for the estimation of blood sugar. This method lacks specificity, due to presence of nonglucose reducing substances in the filtrate. Cuprous oxide, so formed, is treated with phosphomolybdic acid solution and re-oxidation of cuprous ions by oxygen, from the air, is prevented by using a specially designed tube, referred to as Folin-Wu tube. Blue color is formed, whose intensity is compared with the standard.

Folin-Wu method is based on the reducing properties of sugars but over estimates the true blood glucose value by 5–20%. This is due to the presence of other sugars, such as fructose, and other reducing substances, e.g. glutathione, vitamin C, etc., in the blood.

Preparation of Protein-free Filtrate

Take 3 test tubes and label them as blank (B), standard (S) and test (T). Add distilled water, standard glucose solution, blood and other solutions as shown in Table 23.1.

Table 23.1 Preparation of the test tubes for obtaining protein-free filtrate for the estimation of blood glucose

	B	S	T
Distilled water	3.4 mL	3.2 mL	3.2 mL
Standard (0.1 mg/dL)	—	0.2 mL	—
Blood	—	—	0.2 mL
10% Sodium tungstate	0.3 mL	0.3 mL	0.3 mL
2/3 N H_2SO_4	0.3 mL	0.3 mL	0.3 mL

Mix the contents and centrifuge/filter after 5 min, to obtain protein-free filtrate.

Procedure

Take 3 Folin-Wu tubes and label them as mentioned above. Transfer 2 mL of the filtrate/supernatant, as obtained above, to the correspondingly labeled Folin-Wu tubes. Thereafter, add 2 mL of alkaline-copper reagent to each, mix and keep in a boiling-water bath for 6 min. Cool and add 2 mL of phosphomolybdic acid solution to each tube. Mix the contents and make up the volume to 25 mL mark with distilled water. Mix the contents and read optical density at 420 nm against water.

Calculation

$$\text{Blood sugar (mg/dL)} = \frac{\text{OD}_{\text{T-B}}}{\text{OD}_{\text{S-B}}} \times \begin{matrix}\text{Concentration}\\ \text{of standard}\end{matrix}$$

$$= \frac{T-B}{S-B} \times 100$$

Reference value of blood sugar for fasting venous blood sample by this method is 80–120 mg/dL. Out of this, reducing substances, other than glucose, constitute nearly 20–30 mg/dL of whole blood.

Somogyi-Nelson Method

Principle

For Somogyi-Nelson method, proteins are precipitated by the addition of barium hydroxide [$Ba(OH)_2$] and zinc sulfate ($ZnSO_4$), which, in turn, forms zinc hydroxide and precipitates proteins. Protein-free filtrate is heated with alkaline copper reagent and the reduced copper, so formed, is treated with arsenomolybdate reagent. This, in turn, results in the formation of violet color, absorbance of which is read by a spectrophotometer.

In the method, besides proteins, sulfhydryl compounds, such as glutathione, are removed as zinc salts while the other reducing substances, e.g. uric acid and creatinine, are precipitated and adsorbed on barium sulfate ($BaSO_4$). Thus, the resultant protein-free filtrate is free from the various nonsugar reducing substances.

Reagents

1. 5% zinc sulfate solution
2. 0.3 N barium hydroxide
3. **Alkaline copper reagent:** It is prepared by mixing 25 parts of solution A with 1 part of solution B. It is freshly prepared on the day of use only. The two solutions are separately prepared, as follows:
   - ***Solution A:*** Dissolve 25 g of anhydrous Na_2CO_3, 25 g of Rochelle salt (sodium potassium tartrate), 20 g of Na_2HCO_3 and 200 g of anhydrous Na_2SO_4 in about 800 mL of water, and dilute the contents to 1 L. This solution can be stored at room temperature. If any sediment is observed, filter it before use.
   - ***Solution B:*** 15% $CuSO_4.5H_2O$ containing one to two drops of concentrate H_2SO_4.
4. **Arsenomolybdate reagent:** Dissolve 25 g of ammonium molybdate in 450 mL of water and add 21 mL of concentrate H_2SO_4 to it. The contents are thoroughly mixed. Three grams of disodium orthoarsenate ($Na_2HAsO_4.7H_2O$) is separately dissolved in 25 mL of water and is added, with stirring, to the acidified-molybdate solution. Thereafter, it is kept at 37°C for 24–48 hours and stored in a glass-stoppered, brown-colored bottle.
5. **Standard glucose solution**
   - **Stock solution:** One g of anhydrous glucose is dissolved in 10–15 mL of 0.2% benzoic acid solution and the volume is made to 100 mL (with the benzoic acid solution).
   - **Working standards:** Three working standards are prepared by diluting 0.5, 1.0 and 2.0 mL of the stock solution, each to 100 mL with the benzoic acid solution.

Procedure

Into a test tube containing 3.5 mL of water, add 0.1 mL of blood and mix the contents. Add 0.2 mL of 0.3 N barium hydroxide solution to it. After the mixture turns brown, add 0.2 mL of zinc sulfate solution and mix the contents. After 10–15 min, the mixture is filtered through a Whatman No. 1 filter paper.

In 2 separate test tubes, transfer 1 mL aliquots of the filtrates, and add 1 mL of alkaline copper reagent. The tubes are covered with a glass marble, placed in a boiling water bath for 20 min and then cooled under running water. Thereafter, one mL of arsenomolybdate reagent is added to each tube and the contents are diluted to 25 mL with water. Simultaneously, a standard and a reagent blank are also prepared. Intensity of the color is read at 500 nm.

Calculation

$$\begin{matrix}\text{Blood sugar}\\ \text{(mg/dL)}\end{matrix} = \frac{\begin{matrix}\text{Reading of}\\ \text{unknown}\end{matrix}}{\begin{matrix}\text{Reading of}\\ \text{standard}\end{matrix}} \times \begin{matrix}\text{Concentration}\\ \text{of standard}\end{matrix} \times 4 \times \frac{100}{0.1} \times \frac{1}{100}$$

O-Toluidine Method

Principle

An aldohexose, such as glucose, when heated with O-toluidine in an acidic medium, forms a condensation product glycosylamine and subsequently, a Schiff's base is formed, which has blue-green color.

O-Toluidine Reagent

O-Toluidine reagent contains O-toluidine, glacial acetic acid and thiourea.

Preparation of Protein-Free Filtrate

Take 0.2 mL of blood/serum, in a test tube. Add 1.8 mL of 4% trichloroacetic acid and centrifuge/filter after 10 min.

Procedure

Take 3 test tubes and mark them as blank (B), standard (S) and test (T). Add distilled water, standard, protein-free filtrate and O-toluidine reagent as shown in Table 23.2.

Table 23.2 Preparation of the test tubes for the estimation of blood glucose by O-toluidine method

	B	S	T
Distilled water	0.5 mL	—	—
Standard (mg/dL)	—	0.5 mL	—
Protein-free filtrate	—	—	0.5 mL
O-Toluidine reagent	2.5 mL	2.5 mL	2.5 mL

Mix and keep in a boiling water bath for 10 min. Cool under tap water and read the optical density at 630 nm, against blank.

Calculation

$$\text{Blood glucose (mg/dL)} = \frac{OD_T}{OD_S} \times \begin{array}{l}\text{Concentration}\\ \text{of standard}\end{array}$$

$$= \frac{T}{S} \times 100$$

Glucose Oxidase Method

Principle

Glucose oxidase (GOD) oxidizes the specific substrate, i.e. β-D-glucose to gluconic acid and liberates hydrogen peroxide (H_2O_2). Thereafter, another enzyme peroxidase (POD) acts on hydrogen peroxide to liberate nascent oxygen (O_2), which, in turn, couples with 4-amino antipyrine and phenol to form pink to red colored quinoneimine dye.

The intensity of the color is directly proportional to the concentration of glucose present in plasma. Intensity of the color is measured at 530 nm (green filter) and compared with the standard, which is treated similarly.

$$\beta\text{-D-Glucose} + H_2O \xrightarrow{GOD} \text{Gluconic acid} + H_2O_2$$

$$H_2O_2 + \text{Phenol} + \text{4-Aminoantipyrine} \xrightarrow{POD} \begin{array}{l}\text{Quinoneimine} + H_2O\\ \text{(pink color)}\end{array}$$

Reagents

1. **Phosphate buffer:** pH 7.0
2. **Enzyme reagent:** It contains GOD, POD, 4-amino antipyrine and phenol in phosphate buffer.
3. **Glucose standard solution:** 100 mg/dL.

Procedure

Take three clean and dry test tubes, and label them as test (T), standard (S) and blank (B). Then add the following in each of test tubes, separately, as shown in Table 23.3.

Table 23.3 Preparation of the test tubes for the estimation of blood glucose by the enzymatic (GOD/POD) method

Contents	T	S	B
Distilled water	–	–	20 µL
Blood sample	20 µL	–	–
Glucose standard solution	–	20 µL	–
Enzyme reagent	2 mL	2 mL	2 mL

Mix and incubate at 37°C for 15 min. Take OD at 530 nm.

Calculation

$$\text{Concentration of Glucose (mg/dL)} = \frac{OD_T}{OD_S} \times 100$$

Normal Values

Normal value for glucose, by the methods other than Folin-Wu, varies between 60 and 90 mg/dL of whole blood or 70 and 100 mg/dL of serum or plasma, in adults. In neonates, blood glucose concentration varies between 30 and 60 mg/dL of serum/plasma, which is still lower in premature neonates. Blood glucose concentration increases with age by about 2–3 mg/dL, per decade.

Glucose concentration in the cerebrospinal fluid (CSF) is nearly 60–65% of the serum/plasma level.

Hypoglycemia

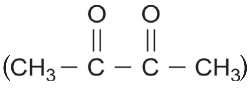
Hypoglycemia (plasma glucose concentration below 40 mg/dL) is a common and potentially dangerous complication of insulin treatment, particularly, in tightly controlled diabetics. Insulinoma (benign adenoma of pancreatic islets) can also cause hypoglycemia. Estimation of plasma insulin concentration, which is raised during fasting, confirms diagnosis.

BLOOD UREA

It is done by diacetylmonoxime (DAM) method.

Principle

Urea reacts with diacetyl under acidic conditions and forms a colored chromogen, called Diazene yellow.

Diacetyl ($CH_3-\overset{\overset{O}{||}}{C}-\overset{\overset{O}{||}}{C}-CH_3$) is an unstable compound, so we use diacetylmonoxime ($CH_3COC{=}NOHCH_3$).

As the color developed is faint and unstable, so ferric alum or thiosemicarbazide is used to intensify and stabilize the color.

Urea can be estimated in whole blood by preparing protein-free filtrate (PFF).

Preparation of Protein-free Filtrate

Take 0.2 mL of oxalated blood, 1.9 mL of 5% zinc sulfate and 1.9 mL of 0.3 N sodium hydroxide solution in a test tube. Mix and centrifuge for 5 minutes. Supernatant is used as PFF.

Reagents

1. **Diacetyl monoxime solution** (DAM; 2% solution in 2% acetic acid): Add 2 g of the solid DAM to about 60 mL of water in a 100 mL volumetric flask and add 2 mL of glacial acetic acid to it. Shake it to dissolve with slight warming, if necessary, and make the volume to the mark with water.
2. **Standard solution of urea**
   - **Stock solution:** 250 mg/100 mL
   - **Working standard:** Dilute stock standard solution to 1:100, so as to make it to 0.025 mg/mL.
3. **Ferric alum solution:** Dissolve 5 g of ferric ammonium sulfate in 500 mL of distilled water. Thereafter, add 500 mL of O-phosphoric acid and 500 mL of sulfuric acid.

Procedure

Take 3 test tubes and label them as test (T), standard (S) and blank (B). Add protein-free filtrate (PFF), standard solution, distil water and other reagents as shown in Table 23.4.

Table 23.4 Preparation of the test tubes for the estimation of blood urea by diacetylmonoxime (DAM) method

	T	S	B
PFF	2 mL	—	—
Working standard (0.025 mg/mL)	—	2 mL	—
Distilled water	—	—	2 mL
DAM	2 mL	2 mL	2 mL
Ferric alum	2 mL	2 mL	2 mL

Keep all the test tubes in boiling water bath for 5 minutes and take OD at 420 nm against blank.

Calculation

$$\text{Blood urea (mg\%)} = \frac{A_t}{A_s} \times \text{concentration of standard}$$

1 mL of standard solution has 0.025 mg urea

2 mL of standard solution has 0.05 mg urea

As volume of blood in 4 mL of PFF is 0.2 mL, so volume of blood in 2 mL of PFF 0.1 mL.

Therefore, concentration of urea in 0.1 mL of blood =

$$\frac{A_t}{A_s} \times 0.05$$

Accordingly, concentration of urea in 100 mL of blood =

$$\frac{A_t \times 0.05 \times 100}{A_s \times 0.1}, \text{ or}$$

$$\text{Blood urea (mg\%)} = \frac{A_t}{A_s} \times 50$$

Reference interval for blood urea = 15–45 mg%.

Blood urea is important for the assessment of kidney function, since the route of excretion of urea is through the kidneys.

Alterations in Blood Urea Levels

Increased urea level in blood

Increased blood urea level above normal is termed as azotemia. When this is associated with clinical symptoms, it is called uremia. Cause can be divided into:

1. **Pre-renal**
   - Increased breakdown of tissue proteins, e.g. in fever and some toxic states.
   - Dehydration, leading to low perfusion of kidneys and, therefore, decreased filtration of urea, e.g. in cases of:

  - Burn
  - Diarrhea
  - Vomiting
  - Severe hemorrhage
  - Diabetic ketoacidosis.

2. **Renal**
   - Glomerulonephritis—acute and chronic
   - Chronic renal failure
   - Chronic pyelonephritis
   - Poisoning, affecting kidneys
   - Nephrotoxic drugs, e.g. gentamicin, cephaloridine, sulfonamide, etc.
   - Diabetic and gouty nephropathy.
3. **Post-renal**
   - Stone, anywhere in the urinary tract
   - Malignancy
   - Stricture of the urinary tract
   - Prostate hypertrophy in the elderly

Decreased urea level

Decreased urea level in blood indicates liver failure.

SERUM CREATININE

Principle

Creatinine reacts with alkaline picrate to form an orange-red complex. This is known as **Jaffe reaction**. Since many substances present in the red cells contribute to false high value, analysis of plasma or serum is preferred.

Preparation of Protein-free Filtrate

Take 1 mL of serum in a test tube. Add 1 mL, each, of distilled water, sodium tungstate and sulfuric acid (2/3 N). Mix the contents and filter/centrifuge after 10 min, to obtain protein-free filtrate.

Reagents

1. 10% sodium tungstate
2. 2/3 N H_2SO_4
3. Saturated picric acid solution: An excess of picric acid is dissolved in distilled water and occasionally shaken well. The supernatant is decanted and used. If picric acid is not of chemically pure grade, it is re-crystalized after dissolving in boiling glacial acetic acid.
4. Alkaline picrate reagent: Five mL of 10% NaOH is added to 25 mL of the saturated picric acid solution, just before use.
5. Working standard solution containing 20 µg/mL of creatinine.

Procedure

Two mL of plasma or serum is added to 14.0 mL of water in a test tube and mixed. Add 1.0 mL of sodium tungstate solution and 1 mL of H_2SO_4. These solutions are mixed well and allowed to stand for 10 min. Color of the mixture turns brown, if the protein precipitation is complete. The mixture is, thereafter, filtered in a dry container.

When whole blood is used, 2 mL of blood is diluted with 14.0 mL of water, and 2 mL each of sodium tungstate and H_2SO_4 are added to it.

Five mL of the tungstic acid filtrate is transferred to a test tube. Thereafter, add 2.5 mL of the dilute picric acid reagent, mixed and allow to stand at 25°C, exactly for 15 min. Read optical density at 520 nm within the 5 min. For best reproducibility, time interval between the addition of alkaline picrate reagent and the reading in colorimeter should be kept constant for all the tubes.

Various amounts of the solution containing 5 to 20 µg of creatinine per tube, are pipetted out and the volume is made to 2.5 mL in each tube with water. Thereafter, 5.0 mL of alkaline picrate reagent is added to each tube for development of color. Rest of the procedure is same, as for samples.

A reagent blank is prepared by treating 5 mL water with 2.5 mL picric acid. Its OD is taken and the value is deducted from the OD of the sample and the standard, to obtain the actual values.

Take 3 test tubes, and mark them as blank (B), standard (S) and test (T).

Add distilled water, standard, protein-free filtrate and other reagents as shown in Table 23.5.

Table 23.5 Preparation of the test tubes for the estimation of serum creatinine

	B	S	T
Distilled water	4 mL	3 mL	2 mL
Standard creatinine solution (0.01 mg/mL)	—	1 mL	—
Protein-free filtrate	—	—	2 mL
NaOH	1 mL	1 mL	1 mL
Picric acid	1 mL	1 mL	1 mL

Mix the contents and read optical density at 520 nm, after 15 min.

Calculation

$$\text{S. creatinine (mg/dL)} = \frac{OD_T}{OD_S} \times \begin{matrix}\text{Concentration}\\ \text{of standard}\end{matrix}$$

$$= \frac{T}{S} \times 2$$

Normal value for serum (plasma) creatinine is 0.8–1.5 mg/dL. It is slightly increased in elderly individual, due to its decreased excretion. Serum level is lower in women than in men. Serum creatinine is increased in renal failure and obstruction to urine outflow.

SERUM PROTEINS

TOTAL SERUM PROTEINS

Biuret Method

It is referred to as the biuret method, since biuret which is obtained on heating urea, also gives the same color with cupric ions, as proteins. Besides biuret, this reaction can occur with compounds containing at least two peptide bonds. Free amino acids and dipeptides do not give this test. Intensity of the color is directly proportional to the number of peptide bonds that are present in a protein.

Principle

Peptides, containing at least two adjacent peptide bonds (–CONH–), react with cupric ions (Cu^{2+}) in alkaline copper sulfate solution and form violet colored complex, which has absorption maxima at 540 nm.

This complex is formed between Cu^{2+} ions, and the carbonyl oxygen and amide nitrogen of the peptide bond.

Biuret reagent, which is used for the quantitative estimation of protein in serum, is referred to as **quantitative biuret reagent**. It has copper sulfate, sodium potassium tartrate, potassium iodide and sodium hydroxide.

Reagents

1. **Biuret reagent:** Dissolve 4.25 g of potassium sodium tartrate ($KNaC_4H_4O_6.4H_2O$), 1.5 g of cupric sulfate ($CuSO_4.5H_2O$) and 2.5 g of potassium iodide in about 500 mL of distilled water. Thereafter, dissolve 4 g of NaOH in this solution and make up the volume to 1 L with distilled water.
2. **Standard:** Standard protein solution, used for this purpose, may either be pooled normal human serum (standardized by Kjeldahl method) or a solution of pure albumin in normal saline.

Procedure

Take 3 test tubes, and label them as blank (B), standard (S) and test (T). Add distilled water, standard protein solution, serum and biuret reagent as shown in Table 23.6:

Table 23.6 Preparation of the test tubes for the estimation of serum total proteins by biuret method

	B	S	T
Distilled water	2.5 mL	—	2.4 mL
Standard protein solution (280 mg/100 mL)	—	2.5 mL	—
Serum	—	—	0.1 mL
Biuret reagent	3.5 mL	3.5 mL	3.5 mL

Mix the contents and place the tubes for 10 min at 37°C or for 30 minutes at room temperature (if below 37°C). Read optical density of test and standard against blank at 540 nm.

Calculation

Total protein (g/100 mL)

$$= \frac{\text{OD of test}}{\text{OD of standard}} \times \begin{matrix}\text{Concentration}\\ \text{of standard}\end{matrix}$$

$$= \frac{T}{S} \times \frac{2.8 \times 2.5 \times 100}{0.1 \times 1000}$$

$$= \frac{T}{S} \times 7$$

Total protein concentration in a normal serum varies between 6.3 and 8.4 g/dL.

Refractometry

It is a quick alternative to chemical analysis for serum total protein, when a rapid estimation is required. Refractometry is based on the principle of refractive index. Refractive index of water, at 20°C, is 1.33. After a solute is added to water, refractive index value of the solution is increased linearly, proportionately to the concentration of the solute.

Pre-calibrated clinical refractometers for total solids are available commercially, where concentration of protein in the sample is directly expressed in g/dL.

However, large positive errors occur with azotemia and hyperglycemia. Further, when protein concentration is less than 3.5 g/dL, results are not very accurate.

DIFFERENTIAL SERUM PROTEINS

Serum Albumin

Serum albumin is, generally, estimated by dye-binding method.

Certain dyes, such as **bromocresol green** (BCG), bind very specifically to albumin. On addition of the alkali, color of BCG is changed from green to blue, intensity of which is proportional to the amount of albumin present in serum.

Principle

Increase in absorbance of bromocresol green (BCG) dye at 630 nm on binding to serum albumin at pH 4.2 is directly proportional to the concentration of albumin.

Reagents

1. Buffered-dye solution: Dissolve 8.85 g of succinic acid, 108 mg of BCG (sodium salt) and 100 mg of sodium azide in about 950 mL of water. Add 4 mL of 30% Brij-35. Adjust the pH to 4.2 and make up the volume to 1 L with water. It is stable for 4–5 months at room temperature.
2. Standard albumin solution: A pooled serum sample may be used as standard, after the estimation of albumin in it, by Kjeldahl method (bovine serum albumin is unsatisfactory but human serum albumin fractions of highest purity, which are available commercially, may be used).

ALBUMIN : GLOBULIN RATIO

Albumin : globulin ratio (A : G ratio) can be calculated by estimating serum albumin and then subtracting its value from the value of total serum proteins concentration.

Globulins = Total serum proteins – Albumin

$$\text{A : G ratio} = \frac{\text{Concentration of albumin (g/dL)}}{\text{Concentration of globulins (g/dL)}}$$

Normal value for A : G ratio is from 1.5 : 1 to 2.5 : 1.

Procedure

Take 3.0 mL of the buffered-dye solution in a series of test tubes. Add 10 μL of serum sample, vortex and read the absorbance immediately (within 30 sec) at 630 nm against blank (dye solution). Analyse, in duplicate, and calculate albumin content of the sample, by comparison with the standard which is also run simultaneously.

Electrophoresis

Electrophoresis is a technique for the separation of the charged particles in an electric field. A charged particle when placed in an electric field, migrates towards anode or cathode depending on the net charge carried by the particles.

Different molecules migrate at different speeds depending upon their charge, particle size and shape. Their movement occurs in a liquid medium (a buffer), supported on an inert material, like filter paper, cellulose acetate strip, agar gel, starch gel or polyacrylamide gel (Chapter 38).

SERUM URIC ACID

Uric acid is the end product of catabolism of purine bases present in the nucleoprotein molecules. Therefore, formation of uric acid is principally endogenous but some amount is also formed from purine containing compounds present in food. Thus, serum uric acid level depends, mainly, on the rate of tissue nucleoprotein breakdown and is only marginally affected by diet.

Chemically, uric acid is 2,6,8-trihydroxypurine. Therefore, it can form mono- and disodium salts. These salts are deposited in the joints, in gout, causing symptoms of arthritis.

Renal handling of uric acid is complex and involves four sequential steps:

1. Glomerular filtration of virtually all the uric acid in capillary plasma.
2. Reabsorption, in the proximal convoluted tubule, of about 98 to 100% of filtered uric acid.
3. Subsequent secretion of uric acid into the lumen, in the distal portion of the proximal tubule.
4. Further reabsorption in the distal tubule.

The net urinary excretion of uric acid is 6 to 12% of the amount filtered. The urate pool in the body is about 1.2 g in men and 600 mg in women. pKa of uric acid is 5.75. Above this pH, uric acid exists, chiefly, as urate ions, which is more soluble than uric acid. At pH <5.75, uric acid is predominant.

Collection of Blood

Early morning blood sample should be collected. Purine-rich diet should be avoided in the night just before the day of collection. The blood sample should be estimated as early as possible because bacteria breakdown uric acid on long term storage. Thymol, an antibacterial agent, can be added as preservative.

METHODS OF ESTIMATION

Colorimetric Method

Serum uric acid is estimated by using phosphotungstic acid, called phosphotungstic acid method or Caraway method. It is based on the reducing property of uric acid.

Principle

Uric acid reacts with phosphotungstic acid in the presence of alkali (sodium carbonate) and reduces it to blue colored phosphotungstate complex, the absorbance of which is measured at 700 nm. The color is developed at alkaline pH (pH 10).

Preparation of Protein-free Filtrate (PFF)

Take 8 mL of distilled water, 1 mL of serum, 0.5 mL of 10% sodium tungstate and 0.5 mL of 2/3 N sulfuric acid. Mix properly and wait for 10 minutes. Centrifuge at 4000 rpm for 5 minutes.

Procedure

Take 3 test tubes and label them as T (test), S (standard) and B (blank), and add various reagents as shown in Table 23.7.

Table 23.7 Preparation of the test tubes for the estimation of serum uric acid

	T	S	B
PFF	3 mL	—	—
Working standard (0.01 mg/mL)	—	3 mL	—
Distilled water	—	—	3 mL
14% sodium carbonate	1 mL	1 mL	1 mL

Mix and wait for 5 minutes. Thereafter, add 1 mL of phosphotungstic acid to each tube, mix well and take absorbance after 10 minutes, after setting the zero with blank.

All the reagents should be added in the specified sequence only, to get the accurate results.

Calculations

$$\text{Uric acid (mg\%)} = \frac{A_t}{A_s} \times \text{Conc. of standard}$$

As 1 mL of the standard solution has 0.01 mg of uric acid, hence 3 mL will have = 0.03 mg

Since, 10 mL of PFF has serum 1mL of serum, so 3 mL will have = 0.3 mL of blood

Accordingly, concentration of uric acid in 0.3 mL

$$= \frac{A_t}{A_s} \times 0.03$$

Uric acid concentration in 100 mL of blood will be

$$\frac{A_t \times 0.03}{A_s \times 0.3} \times 100 \text{ or}$$

$$\text{Uric acid (mg\%)} = \frac{A_t}{A_s} \times 10$$

Normal Range

- Females: 2.5–6.2 mg%
- Males: 4.5–7.0 mg%

Sources of Error

1. Precipitation of some amount of uric acid with the proteins, hence, giving low results.
2. Presence of other reducing compounds like glutathione and ergothionine, also add to the color and give false high results. These compounds are mainly present in RBCs, hence, the error can be eliminated by using serum and avoiding the use of the hemolyzed samples.

Enzymatic Methods

1. **Direct uricase method:** Enzyme uricase is used to oxidize uric acid, to form allantoin. The amount of uric acid oxidized is obtained by observing the decrease in absorbance at 293 nm.

$$\text{Uric acid} + 2\,H_2O + O_2 \xrightarrow{\text{Uricase}} \text{Allantoin} + CO_2 + H_2O_2$$

2. **Indirect method:** In this method, H_2O_2 formed in the above reaction is measured colorimetrically using catalase or peroxidase linked reaction. Increase in absorbance at 340 nm gives a direct measurement of uric acid.

   In this method, there is no interference from endogenous compounds and drugs.
3. In another indirect method, increase in oxygen consumption is noted which acts as a direct measure of uricase. But this is not a popular method, yet.

Alterations in Serum Uric Acid Levels

Hyperuricemia

Hyperuricemia associated with gout (serum uric acid level >7 mg/dL in males or >6 mg/dL in females) may be due to:

- Renal disorder leading to decreased excretion in urine

- Increased production of uric acid with:
  - * **Primary causes**
    - – HGPRTase deficiency
    - – PRPP synthetase overactivity
    - – Glucose-6-phosphatase deficiency
    - – Adenosine deaminase deficiency
  - * **Secondary causes**
    - – Polycythemia
    - – Chronic lymphocytic leukemia
    - – Psoriasis

Hypouricemia

Serum uric acid level <2 mg/dL may be due to:

- Enzyme deficiency, e.g. xanthine oxidase
- Renal tubular diseases leading to decreased reabsorption in kidney
- Decreased synthesis, e.g. in liver diseases
- Drugs inhibiting purine synthesis, e.g. 6-mercaptopurine and azathioprine.

SERUM BILIRUBIN

Bilirubin is unstable when exposed to light. It is also unstable at room temperature. Therefore, its estimation should be done as early as possible. Most commonly used method is based on the **van der Bergh reaction** and is referred to as **Malloy and Evelyn method**.

Principle

Bilirubin is estimated quantitatively by means of a diazo reagent. Diazotized sulphanilic acid (sulphanilic acid treated with a mixture of sodium nitrite and hydrochloric acid), in presence of bilirubin forms a purple color compound, referred to as azobilirubin, which is measured colorimetrically. There are two types of the reactions, called direct reaction and indirect reaction.

When the reaction is carried out in an aqueous medium, it is the conjugated bilirubin, which is water-soluble, reacts directly with the diazo reagent. It is called **direct van der Bergh reaction**.

When the reaction is carried out in the presence of methanol, the intramolecular hydrogen bonds are broken and, both conjugated and unconjugated bilirubin reacts, giving the value of total bilirubin. Quantity of the unconjugated bilirubin is obtained after subtracting the value of conjugated bilirubin from the value of total bilirubin.

Reagents

1. **Diazo reagent:** It is prepared fresh by adding 0.3 mL of solution B to 10 mL solution A.
   - ***Solution A:*** Dissolve 1 g of sulphanilic acid in 15 mL of concentrate HCl. Make the volume to 1 liter with distilled water.
   - ***Solution B:*** Weigh 0.5 g of sodium nitrate and dissolve it in small quantity of distilled water and make the volume to 1 liter.
2. Methanol.
3. Hydrochloric acid: 15 mL of concentrate HCl is diluted to 1 liter with distilled water.
4. **Standard bilirubin solution:** Dissolve 11.7 mg of bilirubin in chloroform (200 mmol/L) in an amber color bottle and store at low temperature in dark.

Procedure

For the estimation of total bilirubin, take three test tubes and mark them as test (T), standard (S) and blank (B). Add various reagents as shown in Table 23.8.

Table 23.8 Preparation of the test tubes for the estimation of serum total bilirubin

	T	S	B
Distilled water	1.8 mL	1.8 mL	2.0 mL
Standard solution	—	0.2 mL	—
Serum	0.2 mL	—	—
Diazo reagent	0.5 mL	0.5 mL	—
Hydrochloric acid	—	—	0.5 mL
Methanol	2.5 mL	2.5 mL	2.5 mL

Keep for 30 minutes in dark and read their optical densities at 540 nm.

Concentration of the direct-reacting bilirubin can be determined in a similar way by adding 2.5 mL of water, instead of 2.5 mL of methanol in the test tube.

A standard curve should be prepared by using different concentrations of the standard.

Calculation

$$\text{Total bilirubin (mg/dL)} = \frac{OD_T}{OD_S} \times \begin{array}{c}\text{Concentration}\\ \text{of standard}\end{array}$$

Note: Since bilirubin, as such, is very unstable and is not easily available in the pure form, an artificial standard, containing methyl red, potassium permanganate or pontacyl violet, is commonly used.

- Reference interval: 0.2–0.8 mg%
- Latent jaundice: 0.8–2.0 mg%
- Clinical jaundice: >2 mg%

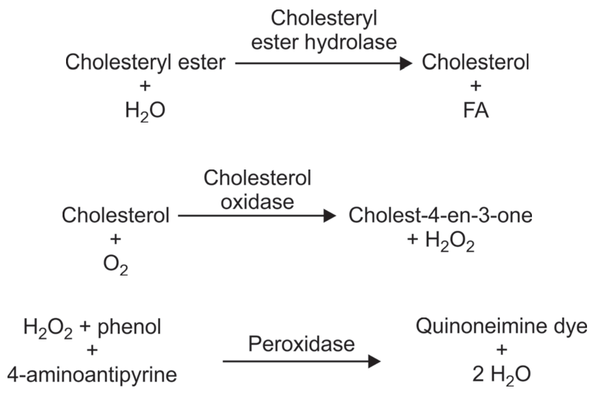
SERUM CHOLESTEROL

ESTIMATION OF TOTAL CHOLESTEROL

1. Enzymatic Method

Principle of the Method

- Cholesterol esters in serum are hydrolyzed by **cholesterol esterase (CE)**. The free cholesterol is then oxidized by **cholesterol oxidase** to the corresponding ketone liberating hydrogen peroxide, which is then converted to water and oxygen by the enzyme **peroxidase**.
- Para-aminophenazone (4-aminophenazone) takes up the oxygen and together with phenol, forms a pink colored quinoneimine dye, which can be measured at 500 nm.
- Free or unesterified cholesterol can be measured by deleting CE from the reagent.



Procedure

- 3–10 µL of serum/plasma + 1000 µL reagent.
- Incubate for 10 min at 20–25°C or 5 min at 37°C and then absorbance is measured at 500 nm.

This reagent contains:

- 4-Aminoantipyrine
- Phenol
- Cholesterol esterase
- Cholesterol oxidase
- Peroxidase
- Sodium azide

Advantages

1. Precise and accurate.
2. Requires only a single, noncorrosive reagent.
3. When reagent-instrument-calibrator system from single manufacturer is used, cholesterol values are accurate within 1–3% of reference values.
4. Requires very low sample volume, 10 µL.
5. No deproteinization is required.
6. Linearity up to 700 mg%.
7. Less time-consuming, only 10 min.
8. No interference by icteric and hemolyzed sera.

Drawbacks

1. Interference from colored compounds or those that compete with oxidation reaction, such as bilirubin, ascorbic acid and Hb.
2. Bilirubin oxidase and dual wavelength readings are used to minimize interference.
3. Enzymatic reagents are not entirely specific for cholesterol because beta-hydroxy sterols and plant sterols can also react but these are present in low concentration in human serum.

Normal Values

• Up to 200 mg/dL	Desirable blood cholesterol
• 200–239 mg/dL	Borderline high
• ≥240 mg/dL	High blood cholesterol

2. Reference Method

Libermann-Burchard Reaction

Commonly used for colorimetric assay.

Principle

Cholesterol is treated with acetic anhydride and concentrate H_2SO_4 in anhydrous conditions. Blue color develops due to formation of monosulfonic acid derivatives of 3,5-diene, resulting from dehydration and oxidation of cholesterol. Absorbance is read at 620 nm.

- Reading is expressed either in terms of molar or mass concentration, which can be interconverted by mg/dL = mmol/L × 38.7.
- This method exhibits approximately 1.6% positive bias compared with isotope dilution mass spectrometry.

Disadvantages

1. Complex
2. Time consuming
3. Partially manual
4. High level of expertise is required for reliable operation.

3. Uranyl Acetate Method

Principle

Uranyl acetate reagent contains uranyl acetate, ferric chloride and glacial acetic acid. Proteins present in serum get precipitated with this reagent. This removes all the interfering substances including bilirubin.

Supernatant is made to react with concentrate sulfuric acid, which acts as a dehydrating agent and forms cholestene polyene carbonium ions which is red in color. Intensity of color is directly proportional to the concentration of cholesterol in given sample.

Procedure

A. **Preparation of protein-free filtrate (PFF):** Take two test tubes and mark them as T (test) and S (standard). Add uranyl acetate reagent, serum and standard as shown in Table 23.9.

Table 23.9 Preparation of the test tubes for preparing protein-free filtrate for the estimation of serum cholesterol by uranyl acetate method

	T	S
Uranyl acetate reagent	4.95 mL	4.95 mL
Serum	0.05 mL	—
Standard (200 mg%)	—	0.05 mL

- Mix properly and wait for 10 min, centrifuge.

B. **Estimation of serum cholesterol:** Take three test tubes and mark them as T (test), S (standard) and B (blank). Add PFF, standard solution and other reagents as shown in Table 23.10.

Table 23.10 Preparation of the test tubes for the estimation of serum cholesterol by uranyl acetate method

	T	S	B
PFF	3 mL	—	—
Standard	—	3 mL	—
Uranyl acetate reagent	—	—	3 mL
H_2SO_4 reagent	1 mL	1 mL	1 mL

- Mix properly. Wait for 15 min.
- Take OD at 560 nm.
- H_2SO_4 reagent contains H_2SO_4, $FeSO_4$ and glacial acetic acid.
- Cholesterol = $\frac{OD_t}{OD_s} \times 200$ mg%

Precautions

- Glassware should be absolutely clean and dry.
- Bigger size test tubes are taken so that proper mixing can occur.
- Uranyl acetate is radioactive, so, should be handled carefully.

4. Zak-Zlatkis Method

Cholesterol reacts with ferric chloride converting ferric ions to ferrous ions. Concentration of ferrous ions is directly proportional to the concentration of cholesterol.

- This method can estimate free as well as esterified cholesterol.
- Digitoxin is used to extract free cholesterol.
- Add digitoxin to diluted serum, centrifuge – supernatant will have esterified, and precipitate contains free cholesterol.

CHLORIDE

Principle

The PFF of the specimen is titrated with mercuric nitrate solution in the presence of diphenylcarbazone as the indicator. The free mercuric ions combine with the chloride ions to form soluble but nonionized mercuric chloride:

$$2Cl^- + Hg^{2+} \rightarrow HgCl_2$$

After all chloride ions have reacted with mercuric ions, any excess mercuric ions combine with the indicator to form blue violet colored complex. Color change of the reaction mixture is considered as the end point of the titration.

Reagents

1. **Mercuric nitrate reagent:** Dissolve 2.93 g of mercuric nitrate in 800 mL of water. Add 20 mL of 2 N nitric acid and make up to 1 L. It is stable at room temperature in amber-colored bottle.
2. **Diphenylcarbazone indicator:** 100 mg/dL in 95% (v/v) ethanol. It is stable at 2–8°C in amber-colored bottle.
3. **Chloride standard (100 mEq/L):** It is prepared by dissolving 5.85 g NaCl in 1 L of distilled water. It is stable at 2–8°C.

Procedure

Preparation of protein-free filtrate

In a centrifuge tube, pipette 4 mL distilled water, 0.5 mL serum, 0.25 mL of 2/3 N H_2SO_4 and 0.25 mL of

10 g/dL sodium tungstate. Mix thoroughly and centrifuge at 3000 rpm for 10 minutes.

Method of Estimation

1. Pipette in a test tube 2 mL of PFF.
2. Add one drop of indicator.
3. Titrate against mercuric nitrate reagent (end point is colorless to violet blue).
4. Note the titration reading (*X* mL).
5. Dilute standard 1 : 10 with distilled water.
6. Pipette 2 mL of dilute standard in a test tube and titrate it against mercuric nitrate reagent, as above.
7. Note the titration reading (*Y* mL).

Calculation

$$\text{Serum chloride (mEq/L)} = \frac{X}{Y} \times 100$$

Reference range for serum chloride is 95–105 mEq/L.

Determination of Urine Chloride

Principle and method is the same as described above for serum, but we use undiluted urine sample. Also, there is no need to prepare PFF.

Average 24 hours excretion is 110–250 mEq.

Determination of Chloride in CSF

Principle and method is the same as described above for serum and urine but we use undiluted CSF sample. Also, there is no need to prepare PFF.

Add 1.8 mL of distilled water and 0.2 mL of undiluted specimen (urine, CSF or any other body fluid) and proceed from step 2 to 4, mentioned above. For the standard tube, add 1.8 mL of deionised water and 0.2 mL of chloride standard (100 mEq/L), and perform step number 6 and 7.

Reference range for chloride in CSF is 700–750 mg/dL.

Alterations in serum chloride levels

Low chloride values are observed in

1. Prolonged vomiting
2. Burns
3. Salt-losing renal disease
4. Overhydration

High chloride values are observed in

1. Dehydration
2. Renal tubular disease
3. Congestive heart failure

PRACTICAL EXERCISES

1. Estimation of blood urea
2. Estimation of serum creatinine
3. Estimation of total proteins in serum
4. Estimation of serum uric acid
5. Estimation of serum bilirubin
6. Estimation of serum cholesterol
7. Estimation of serum chloride

Important Questions

1. What is a colorimeter? Describe various components of a colorimeter with diagram.
2. Write notes on:
   i. Spectrophotometer
   ii. Standard calibration curve

Chapter 24

Electrolytes

An electrolyte is a substance that ionizes when dissolved in suitable ionizing solvents such as water. This includes most soluble salts, acids and bases. Chemically, electrolytes are substances that become ions in solution and acquire the capacity to conduct electricity.

Electrolytes are present in the human body and the balance of the electrolytes in our body is essential for normal function of cells and organs. Common electrolytes that are measured in blood include sodium, potassium and lithium. Their functions are described in Table 24.1.

Table 24.1 Functions of major electrolytes

Sodium
1. Regulation of osmotic pressure of extracellular fluid.
2. Regulation of acid–base balance of extracellular fluid.
3. GI and renal absorption of monosaccharides and amino acids.
4. Maintenance of neuromuscular irritability and excitability.
5. Maintenance of blood viscosity.
6. Cofactor for Na^+/K^+-ATPase.
Potassium
1. Regulation of intracellular osmotic pressure.
2. Regulation of intracellular acid–base balance.
3. GI secretions.
4. Maintenance of neuromuscular irritability and excitability.
5. Storage of proteins and glycogen.
6. Cofactor for enzymes, e.g. Na^+/K^+-ATPase, pyruvate kinase, etc.
Lithium
1. Most widely used medication for treating bipolar disorders.
2. Helps reduce the severity and frequency of mania.
3. Significantly reduces suicide risk.

SODIUM

Adult human body contains about 100 g of sodium, as sodium ion (Na^+). Nearly half of it is found in the extracellular fluid while small amount is intracellular. A small amount is also found in bones.

Na^+ is the main extracellular cation, which exists in the body in association with several anions such as chloride, bicarbonate, phosphates, lactate and proteins. It is the Na^+, K^+-ATPase, which maintains high extracellular concentration of Na^+.

Sodium is freely filtered by the glomeruli. About 70% of the filtered sodium is reabsorbed in the proximal tubule, 15% in the loop of Henle, 5% in the distal convoluted tubule while the remaining 10% by the collecting duct.

Active reabsorption of Na^+ in the proximal tubule also results in the passive reabsorption of Cl^- and HCO_3^- along with water. Reabsorption of Cl^- and HCO_3^- with Na^+ helps to maintain electrical neutrality of the extracellular fluid while reabsorption of water ensures normal osmotic pressure.

Biological Functions

1. **Regulation of osmotic pressure:** Sodium plays a dominant role in the maintenance of osmotic pressure of different body fluids and protects body against excessive fluid loss.
2. **Regulation of acid–base balance:** Sodium salts with the corresponding weak acids form chief buffer system of the extracellular fluid and, thus, play an important role in the regulation of pH of the body fluids.
3. **Absorption of monosaccharides and amino acids:** Na^+ also plays an important role in the absorption of monosaccharides as well as amino acids from the small intestine.

4. **Nerve transmission:** Sodium is also important in the maintenance of neuromuscular irritability and excitability.
5. **Maintenance of blood viscosity:** Sodium is also important in the maintenance of blood viscosity.
6. **Electrolytes and water balance:** Sodium is also important in the maintenance of water and electrolytes balance.
7. Na^+ also acts as **a cofactor** for some enzymes, such as Na^+, K^+-ATPase.

Requirements

Daily requirement of sodium (as sodium chloride) for a normal adult varies from 5 to 15 g.

Sources

Most important source of sodium is the common salt. Meat, sea foods, milk, cheese and bread are good sources of sodium. Plant foods are low in sodium.

Alterations in Sodium Homeostasis

Hyponatremia

It is defined as plasma Na^+ concentration **<135 mEq/L**. It is the most common disorder of body fluid and electrolytes balance. Most often it reflects a problem of too much water, not too little Na^+, in the plasma. Drinking large quantities of water rarely causes hyponatremia because of the large capacity of the kidney to excrete dilute urine. If, however, plasma antidiuretic hormone (ADH) is not decreased when plasma osmolality is decreased or if the ability of the kidney to dilute the urine is impaired, hyponatremia may develop even with a normal water intake.

Because Na^+ is the major solute in the plasma, hyponatremia is usually associated with hypo-osmolality. However, hyponatremia may also occur with a normal or even elevated plasma osmolality.

General symptoms include muscle cramp, lethargy, fatigue, disorientation, headache, anorexia, nausea, agitation, hypothermia, seizures and coma. These symptoms, mainly neurological, are a consequence of the swelling of brain cells, as plasma osmolality falls. Excessive brain swelling may be fatal or may cause permanent damage.

Hypernatremia

Hypernatremia is defined as plasma Na^+ concentration **>150 mEq/L**. It is **always hyperosmolar** and is characterized by edema, azotemia and anemia.

Accumulation of Na^+ in the body has been shown to result in a concomitant rise in blood pressure, referred to as **essential hypertension**. Symptoms are primarily neurological which include tremors, irritability, confusion and coma.

POTASSIUM

Potassium (K^+) is the chief cation of the intracellular fluid. Maintenance of its normal level is essential for life. Potassium is freely filtered at the glomeruli.

Biological Functions

1. Potassium helps to maintain normal osmotic pressure of different body fluids and, thus, **maintains water and electrolytes balance**.
2. Potassium salts also form buffer system of the intracellular fluid and, thus, play an important role in the **regulation of pH of the body fluids**.
3. It is also important in the **maintenance of normal neuromuscular irritability and excitability**.
4. Bases in the alkaline digestive juices, such as pancreatic juice and bile, contain **potassium salts**.
5. **Storage of cell protein** and glycogen requires potassium.
6. Potassium also acts as a **cofactor for several enzymes**, such as pyruvate kinase, Na^+, K^+-ATPase, etc.

Requirements

Normal daily intake of potassium for a normal adult has been recommended as 4 g.

Sources

Potassium is widely distributed in plant and animal sources as chloride, phosphate and carbonate salts of inorganic and organic acids and proteins.

Coffee, cocoa, dried beans, wheat bran, most of the vegetables such as potato and tomato, dairy products, fish, eggs, meat, and fruits like banana, peach and orange are good sources of potassium.

Alterations in Potassium Homeostasis

Disorders of acid–base balance, water balance and administration of some drugs are often accompanied by alterations in potassium balance: Either potassium deficit (hypokalemia) or excess (hyperkalemia).

Hypokalemia

Hypokalemia is defined as serum potassium concentration **<3.5 mEq/L**. It is seen in individuals taking potassium losing diuretics (carbonic anhydrase inhibitors, loop diuretics and thiazides), those with severe liver disease, gastrointestinal losses and metabolic alkalosis. Hyperaldosteronism can cause excess excretion of potassium accompanied by increased retention of Na^+ which leads to depletion of plasma K^+. Prolonged hypokalemia, in turn, further causes damage to the kidney.

Both cardiac and skeletal muscles are affected by hypokalemia. Deficiency symptoms include muscular weakness, mental apathy, cardiac arrhythmias (irregular heart beat), tachycardia (heart beat more rapid than usual), hypotension, paralysis, bone fragility, etc.

Hyperkalemia

Hyperkalemia is defined as serum potassium concentration **>5.5 mEq/L**. Hyperkalemia arises when K^+ excretion by the kidney is depressed such as in acute renal failure. Acidosis causes leakage of K^+ from the cell which may increase plasma K^+ while leaving the cells depleted of K^+. Potassium-sparing diuretics may lead to hyperkalemia. Early signs of cardiac damage include arrhythmia followed by a slowing of the heart rate, ventricular fibrillation and arrest in diastole.

Hyperkalemia may lead to CNS depression, mental confusion, numbness or tingling of extremities, and weakness of respiratory muscles resulting in difficulty in breathing and speech. High concentration of potassium is also toxic to the muscle and may cause paralysis.

LITHIUM

Lithium is a trace element in several biological materials. Its normal serum concentration is 0.003 mEq/L. As a mood stabilizer, lithium is mainly used in the treatment of mania, in bipolar disorder. It probably produces the effect by interacting with the transport of monovalent or divalent cations in neurons. It may also interact with nitric oxide (NO) signalling pathway in the CNS. Lithium concentration in the serum of patients undergoing therapy remains in the range of 0.5 to 1.5 mEq/L. Concentration above 2 mEq/L can produce toxic effects.

Blood samples for monitoring lithium treatment should be taken at least 12 hours after the previous dose (may be up to a week). Patients on lithium therapy should also be monitored for thyroid and kidney functions, as it interferes with the regulation of sodium and water levels in the body. Toxic effects of lithium include ataxia, tremor, convulsions, dysarthria, nystagmus and renal impairment. Lithium toxicity is often compounded with sodium depletion.

ESTIMATION OF SODIUM, POTASSIUM AND LITHIUM

Sodium (Na^+), potassium (K^+) and lithium (Li) can be estimated by flame photometry and ion select electrode (ISE).

FLAME PHOTOMETRY

Flame photometer is a device used in inorganic chemical analysis to determine the concentration of certain metal ions, such as sodium, potassium, lithium and calcium. Group 1 and group 2 metals are quite sensitive to flame photometry due to their low excitation energies (Fig. 24.1).

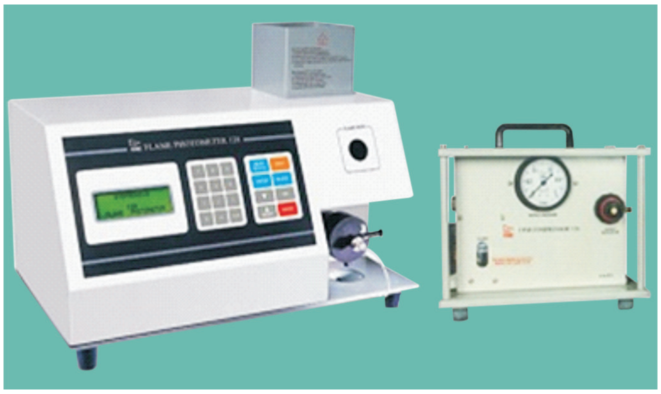


Fig. 24.1: Flame photometer

In principle, it is a controlled flame test with the intensity of the flame color quantified by photoelectric circuitry. The intensity of the color will depend on the energy that had been absorbed by the atoms that was sufficient to vaporise them.

The sample is introduced to the flame at a constant rate. Filters select which colors the photometer detects and exclude the influence of other ions. Before use, the device requires calibration with a series of standard solutions of the ion to be tested.

Flame photometry is crude but a cheap process where emission of radiation by a neutral atom is measured. Since the neutral atoms are obtained by introduction of sample into flame, hence, the name flame photometry. Since radiation is emitted, it is also called flame-emission spectroscopy.

Working Principle of Flame Photometer

When a solution of metallic salt is sprayed as fine droplets into a flame, due to heat of the flame, the droplets dry, leaving a fine residue of salt. This fine residue converts them into neutral atoms. Due to the thermal energy of the flame, the atoms get excited and thereafter return to ground state. In this process of return to ground state, exited atoms emit radiation of specific wavelength. This wavelength of radiation emitted is specific for every element.

The process is simple and applicable to a few elements of group IA and group IIA, like Li, Na, K, Ca and Mg, which are, generally, analyzed by flame photometry. The radiation emitted in the process is of specific wavelength, like for sodium (Na) 589 nm yellow radiation, for potassium 767 nm range radiation, etc.

Components of a Flame Photometer

Burner

This is a part which produces excited atoms. Here the sample solution is sprayed into fuel and oxidant combination. A homogenous flame of stable intensity is produced. There are different types of burners, like total consumption burner, laminar flow and Mecker burner.

Fuel and oxidants are required to produce flame, such that the sample converts to neutral atoms, and get excited by heat energy. The temperature of flame should be stable and also ideal. If the temperature is high, the elements in sample convert into ions instead of neutral atoms. If it is too low, atoms may not go to exited state. So, a combination of fuel and oxidants is used, such that there is desired temperature.

Monochromators

Filters and monochromators are needed to isolate the light of specific wavelength from remaining light of the flame. For this purpose, simple filters are sufficient, as we study only few elements, like Ca, Na, K and Li. So, a filter wheel with filter for each element is taken. When a particular element is analyzed, the particular filter is used so that it filters all other wavelengths.

Detector

Flame photometric detector is similar to that used in spectrophotometry. The emitted radiation is in the visible region, i.e. 400 to 700 nm. Further the radiation is specific for each element, so simple detectors are sufficient for the purpose, like photovoltaic cells, phototubes, etc.

Recorders and display

These are the devices to read out the recording from detectors (Fig. 24.2).

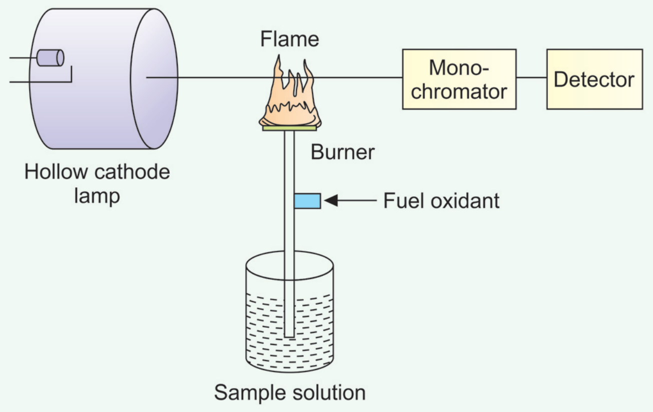


Fig. 24.2: Components of a flame photometer

Limitations of Flame Photometry

Unlike other spectroscopy methods, flame photometry finds little use in research and analysis. This is due to:

- Limited number of elements that can be analyzed. Whereas flame photometry is best suited for the determination of elements, such as sodium, potassium and calcium, the sensitivity of this technique for other elements is rather poor. Moreover, reproducibility of reading is dependent on the temperature of the flame.
- The sample requires to be introduced as solution into fine droplets. Since many metallic salts and other compounds are insoluble in common solvents, hence, they cannot be analyzed by this method.
- Since sample is volatilized, if small amount of sample is present, it is tough to analyze by this method, as some of it goes waste by vaporization.
- Further, during solubilization with solvents other impurities might mix up with sample and may lead to error in the spectra observed.

Estimation of Sodium and Potassium by Flame Photometer

Principle

The sample in solution is introduced in the form of a fine continuous spray into a nonluminous gas flame. The emitted light, characteristic for the ion being analyzed, is isolated and focused on a photoelectric cell and the current intensity is measured on a suitable meter.

Reagents

1. Isotonic glucose solution: 5.9 g glucose in 100 mL water.
2. Standard sodium solution: Prepare a stock standard solution containing 100 mEq of sodium/L by

dissolving 5.85 g of dried NaCl in glass distilled water and dilute it to 1 L. Working standards are prepared by taking 10–16 mL of the stock solution and making up the volume to 1 L. These standards represent 100–160 mEq of Na per L at 1 : 100 dilutions. A further 1 : 5 dilution is employed for all the working standard solutions so that the dilution factor for Na is 500.

3. Standard potassium solution: Prepare a stock standard solution containing 100 mEq of K per L, prepared by taking 15–35 mL of stock standard and diluting it to 1 L. These standards represent 3–7 mEq K, at 1 in 20 dilutions.

Procedure

Plasma: One mL plasma (or serum) is made to 20 mL and aliquots taken for K determination. Dilution factor for this is 20. One mL of this diluted plasma is further diluted to 25 mL and used for Na estimation (dilution factor 500).

Calculation

The sample Na or K is read by interpolation on the standard curves. For plasma (or serum) Na and K, the direct reading from the standard graph can be taken as the dilution factors in the sample and standards are the same.

Estimation of Lithium by Flame Photometer

Sodium and potassium have been found to interfere during assay of lithium by flame photometer.

Reagents

Solutions are to be prepared by using deionized water.

1. Stock TCA: 25 g/100 mL.
2. Working TCA solution: 6.25 g/100 mL.
3. Stock solutions:
   a. Reagent blank: 140 mEq/L of NaCl (8.19 g/L of NaCl), 4.5 mEq/L of KCl (335 mg/L of KCl) and 5 mEq/L of $CaCO_3$ (100 mg/L of $CaCO_3$). Add few mL of concentrate HCl.
   b. Lithium standard (5 mEq/L): 185 mg of $LiCO_3$ is dissolved in minimal volume of concentrate HCl, and volume is made up to 1 L with water.
4. Working lithium standards: 0, 1, 2, 3, 4, 6 and 8 mL of stock lithium standard solution is pipetted into seven different 100 mL volumetric flasks. Twenty mL of the reagent blank (3a) is added along with 20 mL of stock TCA (25 g/100 mL) to each flask before dilution to 100 mL.

   This, in turn, gives a reagent blank and six working solutions of lithium equivalent to 0.25, 0.5, 0.75, 1.0, 1.5 and 2.0 mEq/L.

Serum for lithium estimation is diluted 1 : 4 with TCA before estimation. The fluid is aspirated into the photometer in parallel with standards containing TCA (which also has Na, K and Ca ions, reflecting the physiological range, i.e. 140 mEq/L of Na, 4.5 mEq/L of K and 5 mEq/L of Ca). The results are then derived from the calibration curve plotted between various working solutions of lithium and their emission is recorded.

Ion Select Electrode

Principle

It is based on the measurement of a potential that develops across a selective membrane. The response of the electrochemical cell is therefore based on the interaction between the membrane and the analyte that alters the potential across the membrane.

The electrode consists of a membrane, an internal reference electrolyte of fixed activity, $(a_i)_{internal}$ and an internal reference electrode. The ISE is immersed in a sample solution that contains analyte of some activity, $(a_i)_{sample}$. An external reference electrode is also immersed in a solution (Fig. 24.3).

The internal and external reference electrodes are the two-half cells of the electrochemical cell. The potential measured by the meter is the difference in potential between the external $(E_{ref})_{ext}$ and the internal reference electrodes $(E_{ref})_{int}$ plus the membrane potential (E_{memb}) and the liquid junction potential (E_{lj}) that exists at the junction between the external reference electrode and the sample solution.

$$E_{cell} = E_{ref,ext} - E_{ref,int} + E_{memb} + E_{lj}$$

The glass electrode of a pH meter is a kind of ion select electrode that is sensitive to H^+. Similar potentiometric electrodes have been developed which are responsive to other ions like Na, K, Ca, Li, etc.

PRACTICAL EXERCISES

1. Estimation of sodium and potassium in serum.
2. Estimation of lithium in serum.

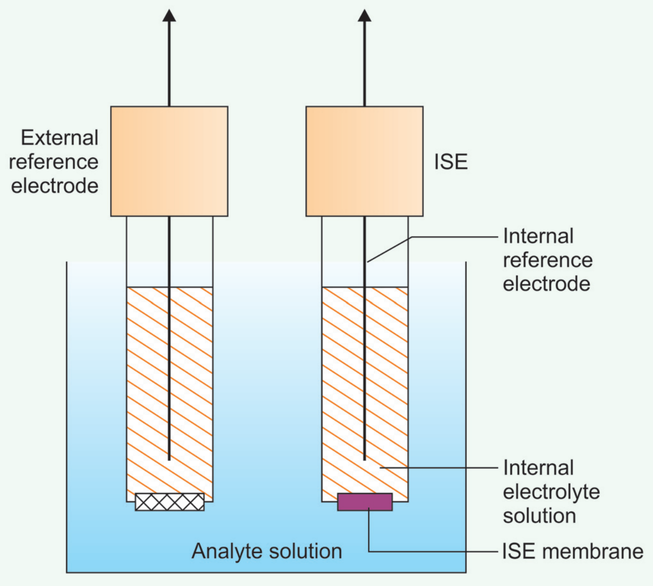

External reference electrode
ISE
Internal reference electrode
Internal electrolyte solution
Analyte solution
ISE membrane

Fig. 24.3: Ion select electrode

Important Questions

1. Describe principle and components of a flame photometer.
2. Write notes on:
   - i. Normal value and biological functions of sodium
   - ii. Normal value and biological functions of potassium
   - iii. Normal value and biological functions of lithium
   - iv. Hyponatremia
   - v. Hypernatremia
   - vi. Hypokalemia
   - vii. Hyperkalemia
   - viii. Flame photometry
   - ix. Ion select electrode

Chapter
25

Statistical Methods

Statistics is the study of the collection, analysis, interpretation, presentation and organization of data. Statistics related to living or human beings is referred to as **biostatistics**.

Data means facts and figures based on scientific evidence. The data can be qualitative or quantitative.

- **Qualitative data** are classified only by counting the units having the same attributes and have no magnitude or size. For example, sex, number of patients cured, attacked by a disease, etc.
- **Quantitative data** means the characteristic or attribute is measurable on a scale, e.g. height, weight, blood pressure, blood glucose, etc.

The science of statistics is used in health service system to evaluate results of services for making decisions in health care delivery.

The hallmark of biostatistics is variations or uncertainties. Variation in characteristic occurs when its value changes from subject to subject, time to time or instrument to instrument.

Statistical Methodologies

Two main statistical methodologies are used in data analysis. These include:

- **Descriptive statistics** which summarizes data from a sample using indexes such as the mean or standard deviation.
- **Inferential statistics** which draws conclusions from the data that are subjected to random variations, e.g. observational errors, sampling variation.

To figure this out for our sample or data set, we have to perform few calculations. First, we will need to find out the mean and variance of our data, and thereafter, we can find out the standard deviation.

- The **mean** is the average of all the data points.
- **Variance** is a measure of how far our data is clustered around the mean.
- **Standard deviation** is found by taking the square root of the variance of sample.

MEAN, MEDIAN AND MODE

There are many kinds of the averages in statistics, but mean, median and mode are the three that are most commonly referred, i.e. they are all measures of central tendency in statistics. In different ways they each tell us what value in a data set is typical or representative of the data set.

Mean

The mean is the average of all the data points. Mean is denoted by the symbol $\bar{x}$.

This is calculated by adding all of the numbers (say x) in the sample and then dividing this figure by how many numbers are there in the sample (n), i.e.

$$\text{Mean} = \frac{\text{Sum of } x \text{ values}}{n \text{ (number of values)}} = \frac{\Sigma x}{n}$$

For example, in the sample of test scores for different values for blood urea

23, 17, 15, 23, 20, 23, 39, 23, 40, 23, 34, 22, 36, 23 and 29

- The first part of calculating a mathematical average or mean is to **add the numbers** (x) in the sample together, i.e.:

  $$23 + 17 + 15 + 23 + 20 + 23 + 39 + 23 + 40 + 23 + 34 + 22 + 36 + 23 + 29 = 390$$

  This is the sum (denoted by the symbol Σ) of all the numbers in the data set or sample.
- Divide the sum by the number (i.e. how many numbers are there in the sample, n). This will provide the average or mean of the data.
  - In the sample of above test scores there are 15 numbers, so $n = 15$.
  - The sum of the test scores in the example is 390.

- – So, divide 390 by 15 to find out the mean, i.e. 390/15 = 26

The mean test score in the sample is 26.

Median

The **median** is the **middle value in the list of numbers**.

The formula for the place to find the median is "([the number of data points] + 1) ÷ 2", but one can also just count in from both the ends of the list, until one meet in the middle.

To find the median, **list all the numbers in numerical order from the smallest to the largest**.

So, first we have to rewrite the list in numerical order:

15, 17, 20, 22, 23, 23, 23, 23, 23,23, 29, 34, 36, 39 and 40

Since there are fifteen numbers in the list, so the middle one will be the (15 + 1) ÷ 2 = 16 ÷ 2 = 8th number, i.e.:

15, 17, 20, 22, 23, 23, 23, **23**, 23, 23, 29, 34, 36, 39 and 40

So, the median is 23.

Note: If there are 2 data values in the middle, the median is the mean of those 2 values.

Mode

The mode is the **number** or numbers **in a data set that occur(s) most frequently**.

For this purpose, count how many times each number occurs in the data set. The mode is the number with the highest tally.

For the data set

23, 17, 15, 23, 20, 23, 39, 23, 40, 23, 34, 22, 36, 23 and 29 the number 23 occurs six times, hence the mode is 23.

Note: If there is more than one mode and if all numbers occur the same number of times, there is no mode. Also, if no number in the list is repeated, then there is no mode for the list.

VARIANCE

The variance is defined as the average of the **squared** differences from the mean. The variance is a figure that represents how far the data in our sample is clustered around the mean. The figure will give us an idea of how far our data is spread out.

Samples with low variance have data that is clustered closely about the mean. On the other hand, samples with high variance have data that is clustered far from the mean. Variance is often used to compare the distribution of two data sets. To calculate the variance, we follow these steps:

- Firstly, work out the **mean** (simple average of the numbers).
- Then for each number, subtract the mean. This will give a figure of how much each data point differs from the mean value.
- Square the above values. This is referred to as the **squared difference**.
- Then work out the **average of the squared differences**.

For example, in our sample of test scores, as mentioned above:

Step 1

Find out the mean.

$$\textbf{Mean} = \frac{\textbf{Sum of } x \textbf{ values}}{n \textbf{ (number of values)}} = \textbf{26}$$

Step 2

To find the variance. For this:

- Subtract the mean value from each of the individual values.
- Now square all the answers you have got from subtraction (Table 25.1).

Table 25.1 Procedure for the calculation of variance

Value (*x*)	Mean – Value	(Mean – Value)^2
23	3	9
17	9	81
15	11	121
23	3	9
20	6	36
23	3	9
39	–13	169
23	3	9
40	–14	196
23	3	9
34	8	64
22	4	16
36	–10	100
23	3	9
29	–3	9
Mean = 26		846

- Add all the squared numbers, which is = 846
- Divide the sum of squares by (n), i.e. 846/15 = 56.4

  Hence, variance = 56.4

STANDARD DEVIATION

Deviation means how far the value is from the normal. In statistics, the **standard deviation** (**SD**; represented by the Greek letter sigma, σ) is a measure, which is used to quantify the amount of variation or dispersion of a set of values. Simply, it is a measure of spread of the numbers, in a set of data, from its mean value. Calculation of the standard deviation tells that how spread out the numbers are in the sample. In addition to expressing the variability of a population, standard deviation is commonly used to measure confidence in statistical conclusions. The reported margin of error is typically about twice the standard deviation, the half-width of a 95% confidence interval.

In science, researchers commonly report the standard deviation of experimental data and only effects that fall much farther than two standard deviations away from what would have been expected are considered statistically significant, normal random error or variation in the measurements is in this way distinguished from causal variation.

A standard deviation close to 0 indicates that the data points tend to be very close to the mean (also called the expected value) of the set, while a high standard deviation indicates that the data points are spread out over a wider range of values.

For a finite set of numbers, the standard deviation is calculated by taking the square root of the average of the squared deviations of the values from their average value.

For example, for the above mentioned 15 data points, which have the mean (average) of 26:

- First, calculate the deviation of each data point from the mean
- Square the result of each
- Calculate the variance, which is the mean of these values.

The standard deviation is equal to the square root of the variance, i.e.:

Standard Deviation (SD or **σ**) = $\sqrt{\mathbf{56.4}}$ or

= 7.51 (to the nearest decimal point)

This formula is valid only if the 15 values, with which we began, form the complete population.

If the values instead were a random sample drawn from some larger population, then it would be divided by 14 (which is $n - 1$), instead of 15 (which is n), in the denominator of the last formula. The quantity, thus obtained would be called the **sample standard deviation**.

Dividing by $n - 1$ gives a better estimate of the population standard deviation for the larger population than dividing by n, which gives a result that is correct for the sample only. This is known as **Bessel's correction**.

COEFFICIENT OF VARIANCE

In statistics, the **coefficient of variance or coefficient of variation** (**CV**), also known as **relative standard deviation** (**RSD**), is defined as the ratio of the standard deviation to the mean. It is widely used in analytical chemistry to express the precision and repeatability of an assay.

The main purpose of finding coefficient of variance is used to study quality assurance by measuring the dispersion of the population data of a probability or frequency distribution, or by determining the content or quality of the sample data of substances. It only uses positive numbers in the calculation and is **expressed in percentage values**.

Therefore, the resultant value of the formula:

$$\mathbf{CV} = \frac{\textbf{Standard deviation } (\sigma)}{\textbf{Mean } (\bar{x})} \times \mathbf{100}$$

How to Calculate Coefficient of Variation?

Follow these below step by step calculation, using above formula to find CV of the sample data:

1. Calculate the mean of the data set.
2. Calculate the sample SD for the data set.
3. Finding the ratio of sample standard deviation to mean, brings the CV of the data set.

Formula to find the sample mean

$$\bar{x} = \frac{\sum x}{n}$$

Formula to calculate sample standard deviation

$$\sigma = \sqrt{\frac{\sum (x-\bar{x})^2}{n-1}}$$

Formula to calculate coefficient of variation

$$\text{CV} = \frac{\sigma}{\bar{x}} \times 100$$

Example

Calculate the coefficient of variance (relative variability) for the above mentioned 15 data points:

Step 1: Calculate mean

Mean = 26

Step 2: Calculate standard deviation

SD = 7.51

Step 3: Calculate coefficient of variance

$$\text{CV} = \frac{\text{Standard deviation}(\sigma)}{\text{Mean}(\bar{x})} \times 100$$

$$= \frac{7.51}{26} \times 100$$

$$= 28.88\%$$

NORMAL DISTRIBUTION

In probability theory, the normal distribution, also known as Gaussian distribution, is a distribution that is observed in many ordinary situations. Normal distributions are important in statistics and are often used to represent real valued random variables whose distributions are not known. For example, if height of students in a class is plotted graphically, a bell-shaped curve is obtained. This is because most of the children will be having an average height while some will be slightly taller or shorter. Only very few children will be very tall or very short. The normal distribution curve is sometimes, informally, called the **bell curve** (Fig. 25.1).

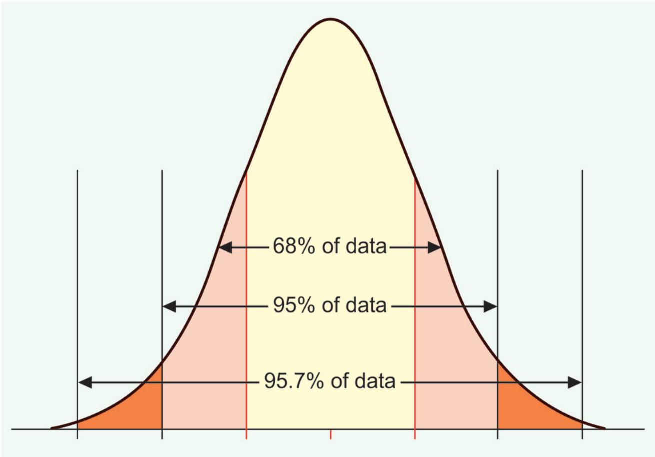


Fig. 25.1: Normal distribution curve

Sixty eight percent of data points fall within one SD of mean, 95% within 2SD and 99.7% within 3SD. In such distribution, mean, median and mode are equal and the area under curve is one. As long as the sample is based on 30 or more observations, the sampling distribution of the mean can be safely assumed to be normal. A normality test is used to determine whether sample data has been drawn from a normally distributed population (within some tolerance). Statistical tests, like Students' t-test, and one way and two-way ANOVA require a normally distributed sample population.

Properties of a Normal Distribution

- The normal curve is symmetrical around the mean
- The mean is at the middle and divides the area into two halves
- The total area under the curve is 1
- It is completely determined by its mean and standard deviation.

PROBABILITY

Probability is the measure of the likelihood that an event will occur. Its value is usually described between 0 to 1. Zero indicates no chances while 1 indicates 100% chance. The higher the probability of an event, the more likely it is that the event will occur. An event with a probability of 0.5 can be considered to have equal odds of occurring or not occurring. For example, the tossing of a fair (unbiased) coin. Since the coin is fair, the two outcomes (head and tail) are both equally probable, i.e. the probability of head equals the probability of tail and since no other outcomes are possible, the probability of either head or tail is 1/2 (which could also be written as 0.5 or 50%). Mathematically, it can be expressed as the number of occurrences of a targeted event divided by the number of occurrences plus the number of failures of occurrences (this is the total of the possible outcomes).

PRACTICAL EXERCISES

1. Calculate mean ± SD for the serum uric acid value for the recent month from your lab.
2. Calculate coefficient of variance for blood glucose values of the previous month of your lab.
3. Plot normal distribution curve for blood urea values of the previous month of your lab.

Important Question

1. Write notes on:
   i. Calculation of mean value
   ii. Standard deviation
   iii. Coefficient of variance
   iv. Probability
   v. Normal distribution curve

Chapter

26

Biochemical Analysis of Kidney and Gallbladder Stones

Nephrolithiasis is a condition associated with the presence of calculi in the urinary tract, i.e. in the renal pelvis, ureter or bladder. Urine contains many minerals and salts. There may be formation of spontaneous crystals, if salt concentrations are very high or provoked by preformed nuclei. Clinical symptoms include severe pain (renal colic), other urinary symptoms, etc.

Diagnosis may be made by radiological investigations like X-ray, urogram or CT scan. Further, investigations include blood, urine and stone analysis.

KIDNEY STONES

TYPES OF KIDNEY STONES

1. **Calcium oxalate** (67%): They are very hard and radio-opaque. Main cause is hyperoxaluria.
2. **Phosphate** (20%): These can be of calcium phosphate or magnesium ammonium phosphate. They are smooth, dirty white, soft and radio-opaque. Main cause is hypercalciuria.
3. **Uric acid and urate** (8%): They are hard, smooth and sometimes rough. They are multiple and radiolucent.
4. Cystine (1–2%): They are multiple and radio-opaque.
5. Mixed stones (2–3%)
6. Miscellaneous: These include:
    - **Calcium carbonate:** They are rare in humans and hard in consistency.
    - **Fibrin:** These are formed from blood clots and may serve as a nidus for other stone formation.
    - **Urostealith:** These are rarely found in bladder. They are soft in consistency and contain fat.

EXAMINATION OF KIDNEY STONES

First step of stone analysis is its gross examination. It includes observing the following points:

- **Number of stones:** Single or multiple
- **Size:** Measure the size with scale
- **Shape:** Shape of stone may be round/oval/irregular
- **Surface:** Stone surface may be smooth/rough/speculated or nodular
- **Color:** Brown, greyish, greyish-white, black, blackish-brown
- **Consistency:** Soft/chalky/firm or hard

Chemical Examination

Wash the stone with distilled water and dry it in oven at 60°C for five hours. Take the stone out and after ensuring complete dryness, it is grounded to fine powder by pestle and mortar. Heat a small amount of the powder in a small crucible/small platinum lid or foil.

- If the **stone is organic in nature**, it burns over entirely or leaves a trace of ash. In such stones, carbonate, phosphate and oxalate are absent.
- If the **stone is inorganic in nature**, little charring may be seen. Observe color of the flame. Cystine stone gives pale blue flame with a rather sharp smell. Fibrin combined with urostealith gives yellow flame while plain fibrin stone releases smell of burnt feathers.

Organic Stones

Test for uric acid

Add 2–3 drops of concentrate nitric acid to a small amount of crushed stone in an evaporating dish or crucible. Evaporate it to dryness by heating on a water bath or a small flame, very carefully. Notice the color of the residue.

If red or yellow residue is obtained, which on cooling changes to purplish-red after the addition of a drop of dilute ammonium hydroxide, this is positive test for uric acid.

If the stone powder dissolves in nitric acid leaving yellow residue, which yields red color on heating or orange color on adding ammonium hydroxide, xanthine stone should be suspected. This indicates negative test for uric acid.

Test for Cystine

Dissolve a small part of stone powder in concentrate nitric acid and let it evaporate off, spontaneously, on a watch glass. Hexagonal crystals of cystine are formed, which can be seen microscopically.

Cystine crystals are insoluble in acetic acid, water, alcohol, ether and acetone but soluble in dilute mineral acids.

Test for Fibrin

Fibrin stone is insoluble in alcohol and ether. It can be dissolved in hot KOH solution from which it is precipitated by acetic acid and liberates hydrogen sulfide.

Inorganic Stones

For the analysis of an inorganic stone, following steps should be performed, sequentially, to test all important components:

- **Step 1:** Add a little dilute hydrochloric acid to a small portion of stone. Liberation of effervescence indicates the presence of carbonate.
- **Step 2:** Warm the solution and filter. Phosphates, cystine and oxalates are present in the dissolved form. Make the pH alkaline with liquid ammonia. This precipitates oxalates and phosphates. Convert the pH to acidic by adding acetic acid. Now the phosphates will dissolve but the oxalates will remain insoluble. This differentiates oxalates from phosphates.
- **Step 3:** Dissolve little stone powder in 1 mL of concentrate nitric acid. Add 1 mL of ammonium molybdate solution. Heat the solution to boiling. Yellow precipitate of ammonium molybdate will appear, if phosphates are present.
- **Step 4:** Heat a little powder with 10% KOH. If ammonia is liberated, it can be detected by its typical pungent smell. This gives an idea about triple phosphate or ammonium urate stone.
- **Step 5:** Phosphates, if present, when tests for NH_3 are negative, are those of Ca^{2+} and Mg^{2+}.
- **Step 6:** Take a small amount of stone powder and dissolve it in dilute HCl. Add ammonia to maintain pH near 5 and add ammonium oxalate solution. If precipitate appears, calcium is present.

Filter this and make the pH alkaline with ammonia. Add solution of potassium hydrogen phosphate. Now, if precipitate settles on standing, magnesium is present.

GALLBLADDER STONES

Gallstones are very rich in cholesterol, bilirubin, calcium and mucoproteins. These stones form when bile is supersaturated with cholesterol or unconjugated bilirubin. Physical appearance of gall stones can be of all shades, from white to quite dark, depending upon the amount of cholesterol and bilirubin, respectively.

Examination of Gallstones

Wash the stone with water, dry it and examine, as follows:

1. **Extract with ether:** Powder the stone and heat some of it with small portion of ether in a test tube, by inserting the tube in warm water. Filter and evaporate the ether. Dissolve the residue in 95% of ethanol and allow it to crystallize. Typical **cholesterol crystals** with notched corners are seen. Thereafter, Libermann Burchard reaction is carried out. Dissolve a little of the residue (obtained on evaporating the ether) in chloroform and add a little of a mixture of acetic anhydride and sulfuric acid (in proportion of 10 mL to 0.1 mL). A dark green colour develops rapidly, indicating the presence of **cholesterol**.
2. **Treat** the residue, remaining after ether extraction, **with 25% dilute HCl**. This, in turn, dissolves the inorganic salts present. Filter and test the filtrate for calcium by making the solution alkaline with ammonia, and add acetic acid and ammonium oxalate solution. If **calcium** is present, white precipitate of Ca-oxalate is seen.
3. **Test the precipitate**, remaining after treatment with HCl, for bile pigments. Wash the material, remaining on the filter paper, with water, dry the paper and extract with warm chloroform. Examine the chloroform extract for **bilirubin** by diazo reagent, used in van der Bergh test.

PRACTICAL EXERCISE

1. Analysis of various types of stones.

Important Question

1. Write notes on:
    - i. Kidney stones
    - ii. Gallbladder stones

Section

2

Analytical Biochemistry and Metabolism

27. Enzymes
28. Vitamins and Coenzymes
29. Metabolism of Carbohydrates
30. Metabolism of Lipids
31. Metabolism of Proteins and Amino Acids
32. Nucleic Acids
33. Metabolism of Hemoglobin
34. Lipid Profile
35. Estimation of Serum Enzymes
36. Spectroscopy
37. Chromatography
38. Electrophoresis

Chapter 27

Enzymes

Enzymes are **organic** molecules, which are produced in living organisms and increase rate of a biochemical reaction without being utilized in the overall process. These are **nondialyzable colloidal particles**, which are **thermolabile proteins** with **highly specific catalytic activity**.

NOMENCLATURE

Enzymes are, usually, named by **the suffix "-ase"** with the **name of the substrate**, or the **catalytic action** of the enzyme, e.g. lipase (which catalyses hydrolysis of lipids), fatty acid synthase (which catalyses synthesis of fatty acids), etc. These names are called **trivial names**. However, while assigning a trivial name to an enzyme, no systematic rules were followed.

CLASSIFICATION

International Union of Biochemistry and Molecular Biology (**IUBMB**) adopted a scheme for rational system of naming each enzyme and suggested their **functional classification**.

According to IUBMB, **each enzyme is assigned four-digit systematic code**, commonly known as **Enzyme Commission number** (**EC number**). **First digit of the code denotes main class of the enzyme**, which is assigned according to type of the chemical reaction it catalyses. **Second digit of the EC number** characterizes **sub-class**, which is based on the nature of the chemical group removed or transferred, or any bond splitted or formed. Its **third digit** indicates **sub-sub-class** and describes detailed subdivision of the sub-class. **Last digit** of the EC number indicates **serial number** of the enzyme in the sub-sub-class. For example, 1.1.1.1 denotes the enzyme (alcohol dehydrogenase), which belongs to class 1, i.e. oxidoreductases. Sub-class 1 of the class 1 (oxidoreductases) includes enzymes, called dehydrogenases. Sub-sub-class 1, of the class 1 (oxidoreductases), of the sub-class 1 (dehydrogenases) includes those dehydrogenases which require NAD^+ as a coenzyme. Accordingly, alcohol dehydrogenase is the first enzyme, which is NAD^+ requiring dehydrogenase, an oxidoreductase.

According to the enzyme commission, enzymes are classified into six classes, which include:

1. Oxidoreductases

Oxidoreductases are involved in the **oxidation and/or reduction of a substrate**, i.e. they catalyze addition of oxygen, removal of hydrogen or removal of electrons. Their sub-classes include dehydrogenases, oxidases, oxygenases, etc. Examples of the class oxidoreductases include alcohol dehydrogenase, xanthine oxidase, glutathione reductase, etc.

2. Transferases

Transferases catalyze **transfer of a particular group from one substrate to another**. On the basis of the group being transferred, they are sub-classified as aminotransferases, phosphotransferases, etc. Glutamate oxaloacetate transaminase, hexokinase, glucose-1-phosphate uridyltransferase are some of the examples of transferases.

3. Hydrolases

Hydrolases **catalyses hydrolysis of a substrate with a molecule of water**. According to nature of the group or bond hydrolyzed, hydrolases are sub-classified as peptidases, glycosidases, etc. Examples of the class hydrolases include glucose-6-phosphatase, amylase, pepsin, etc.

4. Lyases

Lyases catalyze **splitting of a substrate into two products without water**. They are sub-classified on the basis of the linkage splitted, e.g. breakdown of the C–C, C–N or C–S bond, etc. Examples of the class lyases include fumarase, pyruvate decarboxylase, histidase, etc.

5. Isomerases

Isomerases convert one isomeric form of a substrate into other, i.e. they catalyse **isomerisation of the substrate**. Racemases, epimerases and mutases, etc. are sub-classes of isomerases. Examples of the class isomerases include phosphohexose isomerase, phosphoglucomutase, alanine racemase, etc.

6. Ligases

Ligases **catalyse synthesis of a product from two different substrates**. This requires energy, which is coupled to the hydrolysis of a high-energy bond. Its various sub-classes include ligases, which form C–C, C–N or C–S bonds. Examples of the class ligases include glutamine synthetase, acetyl-CoA carboxylase, glutathione synthetase, etc. (Table 27.1).

Table 27.1 Classification of enzymes with examples

Class of enzymes	Examples
Oxidoreductases	Alcohol dehydrogenase, xanthine oxidase, catalase
Transferases	Glutamate pyruvate transaminase, serine hydroxymethyl transferase, hexokinase
Hydrolases	Glucose-6-phosphatase, carboxypeptidase, pepsin
Lyases	Pyruvate decarboxylase, aldolase, histidase
Isomerases	Phosphotriose isomerase, phosphoglucomutase, alanine racemase
Ligases	Glutamine synthetase, acetyl-CoA carboxylase, glutathione synthetase

PROPERTIES OF ENZYMES

Chemical Nature

Most of the enzymes are **protein** in nature. They may have one or more than one polypeptide chains and may be referred to as **oligomeric enzymes** or **multi-enzyme complexes**.

Oligomeric Enzymes

These are **multimeric proteins**, i.e. they have more than one protein subunits, **which are biologically active only when associated with each other**. These multi-subunit enzymes are called **oligomeric enzymes**. For example, phosphorylase, hexokinase and lactate dehydrogenase, each one of which is a tetramer, i.e. they have four subunits. Similarly, phosphofructokinase, fructose-1,6-bisphosphatase and creatine phosphokinase are the dimers, i.e. each one of these enzymes has two protein subunits.

Multi-enzyme Complexes

On the other hand, there are some enzymes, which are single polypeptide chains with several catalytic activities. Due to several enzyme activities associated with a single protein, these enzymes are called **multi-enzyme complexes**. **A multienzyme complex** acts on its substrate and catalyses a series of reactions, e.g. pyruvate dehydrogenase complex, α-ketoglutarate dehydrogenase complex, etc. Each of these enzymes is a multienzyme complex having three different enzyme activities. Fatty acid synthetase is a multi-enzyme complex with seven enzyme activities.

Ribozyme

A ribozyme is a RNA molecule, which exhibits highly substrate-specific catalytic activity. Ribozyme catalyses hydrolysis of phosphodiester bonds in the hnRNA molecule and plays a key role in intron splicing, thereby converting a pre-mRNA to mRNA, e.g. RNase P from *E. coli*.

Proenzymes (Zymogens)

Several enzymes are secreted in the **inactive** form, called zymogen or proenzyme (precursor of the enzyme). Zymogen is converted to its active enzyme (zymase) by some activating agent, such as H^+, other enzyme or the active form of the zymogen. For example, gastric juice secretes **pepsinogen** (a zymogen), which is converted to pepsin by H^+ of the gastric juice. Once pepsin is formed, it acts on pepsinogen and catalyses its conversion to pepsin. This process is referred to as **autocatalysis** (Fig. 27.1).

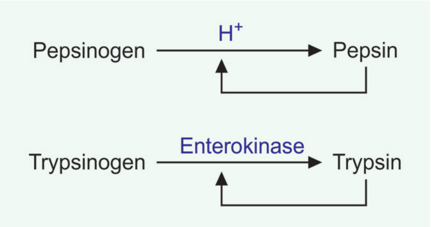


Fig. 27.1: Conversion of a proenzyme to active enzyme

Some of the proenzymes, their activators and active enzymes are given in Table 27.2.

Table 27.2 Some of the proenzymes, their activators and active enzymes

Proenzyme	Activator	Active enzyme
Pepsinogen	H^+, pepsin	Pepsin
Trypsinogen	Trypsin, enterokinase	Trypsin
Chymotrypsinogen	Trypsin	Chymotrypsin
Procarboxypeptidase	Trypsin	Carboxypeptidase
Proelastase	Trypsin	Elastase

Extracellular and Intracellular Enzymes

Zymogens are secreted in the pancreatic juice but exhibit their action in small intestine. Such enzymes, which are produced by cells of a particular tissue but function in some other tissues, are called **extracellular enzymes**.

On the other hand, enzymes which are produced within the cell of a particular tissue and function there only, are called **intracellular enzymes**, e.g. **enzymes of glycolysis**, **citric acid cycle**, **fatty acid synthesis**, etc.

Specificity

Enzymes are highly specific in nature, i.e. they interact with only one or a few substrates and catalyse a specific type of reaction only. The extent of substrate specificity varies from enzyme to enzyme, e.g.:

Absolute Specificity

An enzyme has absolute specificity for its substrate and will not act upon any other substrate, e.g. urease is specific for urea only.

Relative Specificity

An enzyme may be specific either to a particular group or type of bond, e.g. protease (hydrolyzes peptide bond of protein), glycosidase (hydrolyzes glycosidic bond of polysaccharide), esterase (hydrolyzes ester bond of triacylglycerols), etc.

Mechanism of Action

The enzyme forms an intermediate complex with its substrate referred to as **enzyme-substrate complex** (ES).

This reaction can be represented as follows:

$$E + S \rightarrow ES \rightarrow P + E$$

It indicates that the enzyme, first, combines with the substrate and returns to its original form after the reaction is complete.

Various models have been proposed to explain the binding of the substrate with the enzyme.

Most common mechanisms of the binding of the enzyme to its substrate include lock and key model, and induced-fit model:

Lock and Key Model of the Formation of Complex

Complementarity between the enzyme and the substrate is the basis of the lock and key model of enzyme-substrate binding. In this model, during the process of the formation of enzyme-substrate complex, **substrate gets attached to the active site on the enzyme** through some specific groups, **as a key fits into its lock**. This mechanism of enzyme activation was proposed by Emil Fischer and is called **lock and key model**.

Induced-Fit Model

Induced-fit model explains that, though the **substrate-binding sites** on the enzyme are preformed but they **undergo conformational changes after binding of the enzyme with the substrate**.

In the induced-fit model, **substrate induces some changes at the active site of the enzyme**, by changing configuration of protein, so as to attain a shape, where substrate can fit, conveniently. Enzyme-substrate complex is formed by multiple linkages, of the substrate with the enzyme, such as by covalent bonds, hydrogen bonds and electrostatic bonds. This mechanism of enzyme activation was proposed by Koshland, and is called **induced-fit model** (Fig. 27.2).

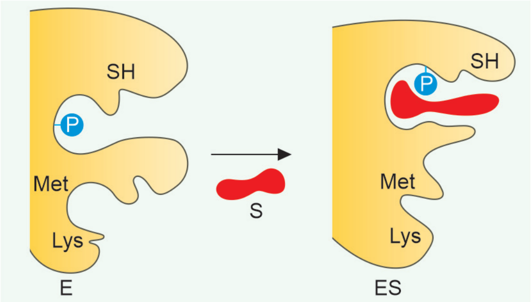


Fig. 27.2: Induced-fit model of the formation of the enzyme-substrate complex

FACTORS AFFECTING ENZYME ACTIVITY

An enzyme exhibits maximum activity only under optimal conditions. Enzyme activity is markedly affected, *in vitro*, by several factors such as temperature, pH, enzyme concentration, concentration of the substrate and the presence of inhibitor.

Temperature

Rate of an enzyme catalyzed reaction increases with increase in temperature of the reaction mixture. It is, however, possible up to certain temperature only, which is called optimum temperature.

Optimum temperature of an enzyme is defined as the temperature at which its activity is maximum. For most of the enzymes, optimum temperature is near body temperature, i.e. 37°C. With **rise in temperature** of the reaction mixture beyond optimum temperature of the enzyme, being protein, **enzyme gets denatured** and loses its biological activity.

Relationship of enzyme activity to a change in temperature is represented by a bell-shaped curve, peak of which represents an **optimum temperature** of the enzyme (Fig. 27.3).

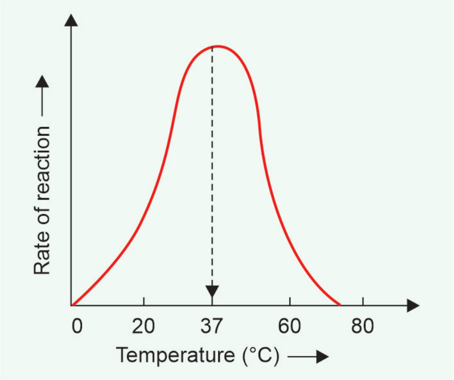


Fig. 27.3: Effect of temperature on enzyme activity

pH

pH of the reaction medium also affects reaction velocity. The pH at which enzyme activity is maximum is defined as its optimum pH. Relationship of enzyme activity to pH is represented by a bell-shaped curve, which has its peak at **optimum pH** of the enzyme (Fig. 27.4).

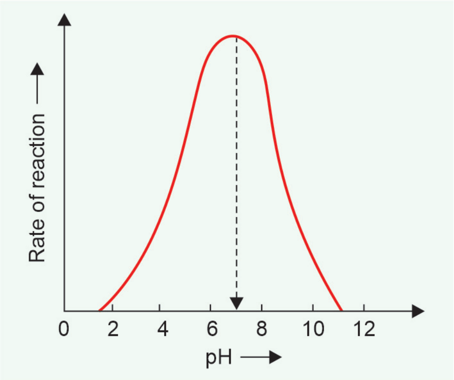


Fig. 27.4: Effect of pH on enzyme activity

Optimum pH of an enzyme depends upon ionization state of the enzyme as well as its substrate. Change in pH affects **ionization state of the enzyme**, thereby **decreasing the number of active sites**. Hence, at extremely low or high pH, enzyme gets denatured. Optimum pH for most of the enzymes is in the neutral range, i.e. around 7.0. However, optimum pH for pepsin is about 2.0 while for the enzymes of the pancreatic juice optimum pH is near 8.0.

Substrate Concentration

With a fixed quantity of enzyme, reaction rate is proportional to concentration of the substrate. This is, however, true only up to a certain point, i.e. the point at which its velocity is maximum (V_{max}). Further increase in substrate concentration, beyond this point, will not increase rate of the reaction (Fig. 27.5).

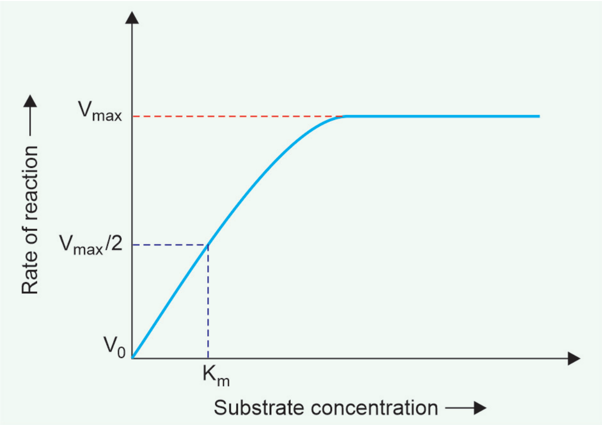


Fig. 27.5: Effect of substrate concentration on enzyme activity

To confirm it, **Michaelis and Menten** derived a relationship between substrate concentration and reaction velocity by an equation, called **Michaelis-Menten equation**, which is as follows:

$$V_0 = \frac{V_{max}[S]}{K_m + [S]}$$

where, V_0 is initial reaction velocity, V_{max} is maximum velocity of the reaction, [S] is concentration of the substrate and K_m is a constant, referred to as **Michaelis constant**.

K_m is defined as the substrate concentration at which reaction velocity is half of the maximum velocity, i.e. $K_m = V_{max}/2$.

Presence of Inhibitor

Phenomenon of decrease in rate of an enzymatic reaction brought about by the addition of a substance other than the substrate (inhibitor) is called enzyme inhibition. An inhibitor may affect enzyme activity either competitively or noncompetitively.

Competitive Inhibitors

A competitive **inhibitor competes with the substrate**, for binding at the active site of the enzyme. **Chemical structure** of a competitive inhibitor, closely, **resembles the substrate**, e.g. malonate (which inhibits the action of succinate dehydrogenase) has structure closely similar to the substrate, i.e. succinate.

A competitive inhibitor forms complex with the enzyme, called **enzyme-inhibitor** (EI) complex, and inhibits action of the enzyme. Binding of the enzyme with the competitive inhibitor, and the substrate, depends upon relative concentrations of the two. It is, usually, a reversible process. By increasing concentration of the substrate, degree of inhibition is reduced. Presence of a competitive inhibitor increases K_m without any change in V_{max} of the enzymatic reaction (Fig. 27.6).

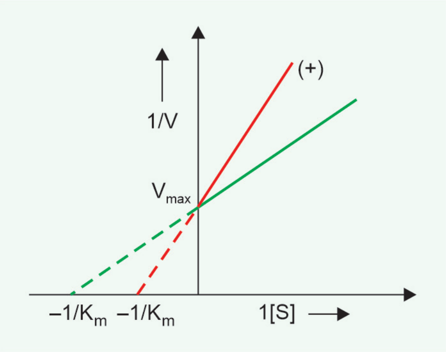


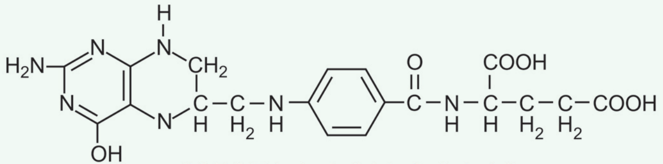
Fig. 27.6: Effect of a competitive inhibitor (+) on enzyme activity

Some of the enzymes, their substrates and competitive inhibitors are given in Table 27.3.

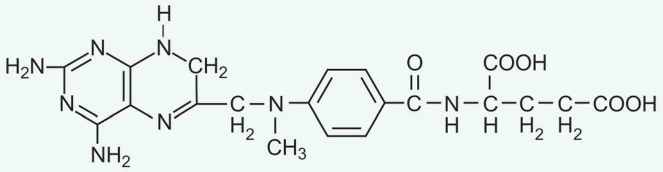
Table 27.3 Some enzymes, their substrates and competitive inhibitors

Enzyme	Substrate	Competitive inhibitor
Succinate dehydrogenase	Succinate	Malonate
Carbonic anhydrase	Carbonate	Acetazolamide
Dihydrofolate reductase	Dihydrofolate	Amethopterin (methotrexate)
Histidase	L-Histidine	D-Histidine
Xanthine oxidase	Hypoxanthine	Allopurinol
HMG-CoA reductase	HMG-CoA	HMG

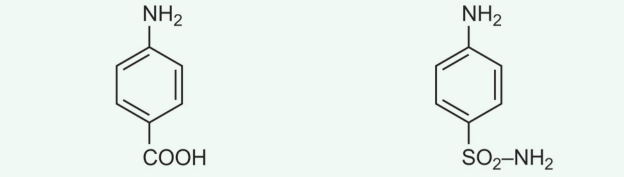
Some drugs act as competitive inhibitors: Several drugs are known to be used as competitive inhibitors. Due to its structural similarity with the substrate, several drugs act as inhibitors of some specific enzymes in the concerned metabolic pathway. For example, **sulfanilamide** (a sulfa drug) is an antibacterial agent and resembles **p-aminobenzoic acid**, structurally. It is used as a **competitive inhibitor** of the enzyme **dihydropteroic acid synthase**, in bacteria. Similarly, **methotrexate**, a **structural analogue of folate, competitively inhibits dihydrofolate reductase**. It is used in the treatment of childhood leukemia (Fig. 27.7).



5,6,7,8-Tetrahydrofolate (substrate)



Methotrexate (inhibitor)



p-Aminobenzoic acid (substrate) Sulphanilamide (inhibitor)

Fig. 27.7: Some drugs (structural analogs) as competitive inhibitors

Noncompetitive Inhibitors

Several **substances react covalently with the functional groups on the active site of the enzyme**. This, in turn, inactivates the enzyme, noncompetitively. For example, enzymes which have free SH-groups in their molecules are inhibited by substances like iodoacetate or *p*-chloromercuro-benzoate, which binds SH-groups, oxidises cysteine molecules and inactivates the enzyme. Heavy metals like Hg^{2+} or Ag^{+} denatures protein and inactivates the enzymes.

This type of inhibition is usually irreversible and makes active sites of the enzyme ineffective. A **noncompetitive inhibitor** may even **bind at a site other than the substrate-binding site**. In such cases, either the enzyme or the enzyme-substrate complex binds with the inhibitor. As a result of it, both, binary (EI) as well as ternary (ESI) complexes may be formed. Under these condition, ESI is converted to product but at a slower rate, thus, a reversible noncompetitive inhibitor **decreases V_{max} but not K_m** (Fig. 27.8).

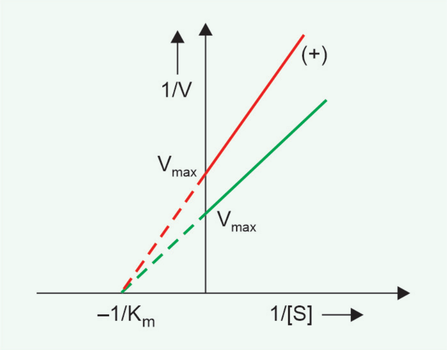


Fig. 27.8: Effect of a noncompetitive inhibitor (+) on enzyme activity

Several drugs are known to be used as noncompetitive inhibitors.

Antimetabolites: Certain derivatives of purines and pyrimidines act as noncompetitive inhibitors. They are called **antimetabolites** and are used as chemotherapeutic agents, e.g. **5-fluorouracil**, an analogue of **thymine**, inhibits **thymidylate synthetase**, **noncompetitively** (Fig. 27.9).

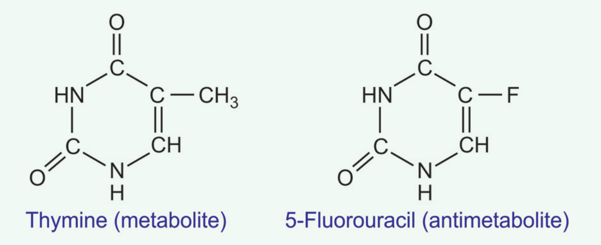


Fig. 27.9: Thymine and its structural analog 5-fluorouracil

UNITS OF ENZYME ACTIVITY

Amount of an enzyme in any sample of a biological fluid, such as serum, or tissue is measured in terms of the rate of reaction catalyzed and is directly proportional to quantity of the enzyme present in the sample. The result is expressed in terms of **enzyme units**. Thus, **unit of an enzyme activity** is a measure of the rate at which a reaction proceeds, e.g. quantity of the substrate consumed or product formed, in mg or micromoles, or change in absorbance, etc., in a unit time (second, minute or hour). In clinical chemistry, result of the enzyme activity is, generally, expressed in a fixed volume, such as per 100 mL (dL) or per liter (L) of serum.

Historically, different types of units have been introduced by different workers, e.g. King-Armstrong units (KA units) and Bodansky units for alkaline and acid phosphatases; Somogyi units for amylase, etc. These units, however, are still in use, in some labs.

International Units

As per Enzyme Commission of the International Union of Biochemistry (IUB) enzyme activity is expressed in terms of **international units** (IU). An IU is defined as quantity of the enzyme which catalyzes reaction using 1 mmol of the substrate or formation of the product that is transformed per minute (1 mmol/min). This is expressed in terms of a fixed volume, e.g. IU/L.

SI Units

To overcome the problem of different units being reported by different laboratories, in different countries, IUB suggested SI units (Systeme Internationale de units, i.e. International System of units). According to it, mole is a measure of the substrate transformed while second is the unit of time. Further, it has been recommended that enzyme activity should be expressed in terms of katal, which is defined as moles/second. It is further recommended that the enzyme concentration should be expressed in terms of katal per liter (kat/L).

APPLICATION OF ENZYMES

Therapeutic Applications of Enzymes

Enzymes are used in the treatment of a number of diseases. For example, u-plasminogen activator (formerly called urokinase), which is extracted from human urine, in the treatment of pulmonary embolism. It stimulates the cascade system for the production of active plasmin, the proteolytic enzyme which digests fibrin, which is the main structural component of blood clot. Some enzymes are also used to restrict the growth of cancer cells by depriving them of essential nutrients, e.g. L-asparaginase. It is used in the treatment of several types of leukemias (Table 27.4).

Table 27.4 Therapeutic applications of enzymes

Enzyme	Therapeutic application
Asparaginase	Acute lymphoblastic leukemia
Streptokinase and urokinase	Lysis of intravascular clot
Lysozyme	Antibiotic
Rhodanese	Cyanide poisoning
Ribonuclease	Antiviral
Lactamase	Pencillin allergy
Streptodornase	DNase (local application)
Papain	Anti-inflammatory
α-Antitrypsin	Emphysema
Pancreatin	Pancreatic insufficiency
Uricase	Gout

Analytical Applications of Enzymes

Enzymes are also important as analytical reagents. Enzyme-based methods of analysis are very sensitive and are usually carried out under mild conditions. Such procedures are widely used in clinical chemistry, e.g. glucose oxidase in the measurement of glucose, cholesterol oxidase for cholesterol, urease for urea, lactate dehydrogenase for lactate and pyruvate, etc. Besides, alcohol dehydrogenase is used to monitor ethanol levels in forensic samples. Enzymes are also used as an alternative to radioisotopes, as markers in immunoassays. Enzyme linked immunoassay (ELISA) is widely used for the determination of several proteins and hormones. Several enzymes, such as horse-radish peroxidase, alkaline phosphatase, glucose-6-phosphate dehydrogenase, α-galactosidase, etc., are used for the immunoassays to estimate HIV antibodies, various therapeutic agents and tumor antigens (Table 27.5).

Table 27.5 Analytical applications of enzymes

Enzyme	Used for the analysis of
Glucose oxidase, peroxidase	Glucose
Cholesterol oxidase, peroxidase	Cholesterol
Lipase	Triacylglycerols
Urease	Urea
Uricase	Uric acid
Horse radish peroxidase, alkaline phosphatase	ELISA
Reverse transcriptase	Polymerase chain reaction (RT-PCR)
Restriction endonuclease	Southern blot, RFLP

Diagnostic Applications of Enzymes

Levels of several enzymes increase in serum, after they are released from a damaged tissue. Some of them also have prognostic significance (Table 27.6).

ISOENZYMES

Isoenzymes (**isozymes**) are **physically distinct forms of an enzyme**, which have **same specificity** but **differ**

Table 27.6 Diagnostic applications of enzymes

Enzyme	Principal source	Clinical situation in which altered
Acid phosphatase	Prostate and RBC	**Increases** in carcinoma of prostate
Alkaline phosphatase	Liver, bone, placenta and kidney	**Increases** in obstructive jaundice, rickets, Paget's disease and hyperparathyroidism
Aldolase	Skeletal muscle and heart	**Increases** in muscular dystrophies and acute liver diseases
Amylase	Salivary glands and pancreas	**Increases** in acute pancreatitis, intestinal obstruction and acute parotitis **Decreases** in acute liver diseases
Ceruloplasmin	Liver	**Increases** in cirrhosis. **Decreases** in Wilson disease
Choline esterase	Liver	**Increases** in nephrotic syndrome and organophosphorus poisoning **Decreases** in acute liver diseases
Creatine phosphokinase	Skeletal muscle, brain and heart	**Increases** in myocardial infarction and muscular dystrophies
Glutamate oxaloacetate transaminase (GOT) and glutamate pyruvate transaminase (GPT)	Liver, skeletal muscle, heart, kidney and RBCs	**Increases** in myocardial infarction, toxic liver cell necrosis, acute liver diseases and muscle diseases
γ-Glutamyltransferase	Liver and kidney	**Increases** in alcoholics and hepatobiliary diseases
Lactate dehydrogenase	Heart, liver, skeletal muscle, RBCs and platelets	**Increases** in acute myocardial infarction, acute hepatitis and muscle disorders
Lipase	Pancreas	**Increases** in acute pancreatitis and pancreatic carcinoma **Decreases** in liver diseases and diabetes mellitus
5′-Nucleotidase	Hepatobiliary tract	**Increases** in liver diseases, obstructive jaundice and hepatic carcinoma
Trypsin	Pancreas	**Increases** in acute pancreatic diseases

from each other with respect to their structure, electrophoretic mobility, immunological properties or source, e.g. lactate dehydrogenase, creatine phosphokinase, alkaline phosphatase, etc.

Lactate Dehydrogenase

Lactate dehydrogenase (**LDH**) **is a tetramer** and has four polypeptide chains (subunits), which may be either H (heart) type and/or M (muscle) type. **LDH**, thus, exists in serum in **five different forms** (isoenzymes), referred to as LDH_1 to LDH_5.

LDH_1 has four polypeptide chains of the H type (4H or H4). LDH_5 has four subunits of the M type (M4). Other forms of LDH are LDH_2 (H3M), LDH_3 (H2M2) and LDH_4 (HM3). LDH_1 and LDH_2 are predominant in myocardium and erythrocytes while LDH_4 and LDH_5 are predominant in the liver and skeletal muscle. LDH_1 concentration increases within l2 to 24 hours of myocardial infarction.

Creatine Phosphokinase

Creatine phosphokinase (**CPK**), also referred to as **creatine kinase** (**CK**), **has three isoenzyme forms**. Each of its **isoenzyme** is a **dimer**, i.e. it is composed of two subunits, which may either be of M (muscle) type or of B (brain) type. Hence, it has three isoenzymes. These are referred to as CPK_1 (BB), which is mainly found in brain; CPK_2 (MB) in myocardium and CPK_3 (MM) in skeletal muscle. Normally, CPK_2 is present in small amount in serum but its level increases within 4 hours and reaches to a maximum value within 1 day of myocardial infarction.

ENZYME PATTERN IN DISEASES

Myocardial infarction (MI)

- Measurement of creatine phosphokinase (**CPK** or **CK**) in serum is of significance in acute MI. Its level starts to rise within 3 to 6 hours of infarction. Estimation of **CK-MB** (CK2) isozyme is most useful marker in the diagnosis of MI.
- **Cardiac troponins** (**troponin I** and **troponin T**) levels rise within 4–6 hours of MI.
- Serum levels of aspartate aminotransferase (AST, also called glutamate oxaloacetate transaminase or GOT) and alanine aminotransferase (ALT, also called glutamate pyruvate transaminase or GPT) are increased in MI. In routine, these enzymes are also called **SGOT** and **SGPT**. Comparatively, AST activity is increased more than ALT in MI. It is due to the reason that concentration of AST is high in cardiac muscle. Peak level of AST is reached after 18 to 24 hours of MI and return towards normal by the fourth or fifth day.
- LDH also has a diagnostic significance in MI. Total LDH as well as **LDH_1** (H4) levels are significantly increased in MI. LDH_1 becomes greater than LDH_2 between 12 and 24 hours following myocardial infarction with peak after 48 to 72 hours. Its level returns towards normal after 6 to 10 days.

Liver Diseases

- In viral hepatitis and other forms of a liver disease **ALT** and **AST** levels are increased even before the clinical signs and symptoms of the disease appear. Levels of both the enzymes may reach values up to 100 folds of the upper normal limits. While their peak levels are seen between seven and twelve days of viral hepatitis, their activities gradually decrease and reach towards normal between three to five weeks. Although serum levels of both the enzymes are elevated, ALT is more liver specific.
- **Alkaline phosphatase** (ALP) is markedly increased in obstructive liver disease.
- **Gamma-glutamyl transpeptidase** (also called γ-glutamyl transferase or γ-GT) is of special significance in detecting an alcohol-induced liver disease. Levels are significantly increased in patients with alcoholic cirrhosis as well as in people who are heavy drinkers.

Muscle Diseases

- **CPK** or **CK** (creatine kinase), particularly CK3 (MM), activity is markedly elevated in a muscle disease.
- **SGOT**, and occasionally **SGPT**, levels are elevated in progressive muscular dystrophy.
- LDH5 (M4) levels are increased in a muscle disease.
- Aldolase level is an early, but nonspecific, marker of a muscle disease.

Cancer

- Five to tenfold rise occurs in **SGOT and SGPT** in patients with metastatic carcinoma of the liver.
- **Acid phosphatase** (ACP) activity is raised in prostate cancer.

Important Questions

1. What are enzymes? Classify them. Discuss factors affecting enzyme activity.
2. What are isoenzymes? Give examples and their diagnostic significance.
3. Give brief account of factors affecting enzyme activity.
4. Discuss briefly:
    i. Various types of enzyme inhibition, giving examples.
    ii. Therapeutic applications of enzymes
    iii. Analytical applications of enzymes
5. Write notes on:
    i. Diagnostic applications of enzymes.
    ii. Noncompetitive inhibitors
    iii. Proenzymes
    iv. Competitive inhibitors
    v. Enzyme specificity
    vi. Ribozymes

Chapter 28

Vitamins and Coenzymes

Vitamins are organic constituents of food called **protective accessory food factors** since these are essential for life and well being. Vitamins, in general, are not synthesized by the body and have to be supplied in diet, though in small quantities.

CLASSIFICATION OF VITAMINS

Vitamins are classified into two groups, referred to as fat soluble vitamins and water soluble vitamins.

WATER SOLUBLE VITAMINS

Water soluble vitamins include **vitamin B complex** and **vitamin C**.

Typical deficiency symptoms for most of the water soluble vitamins, particularly those of vitamin B complex, include dermatitis, glossitis, cheilosis and diarrhea besides some of the neurological symptoms such as peripheral neuropathy and malaise.

VITAMIN B COMPLEX

Vitamin B complex includes a group of compounds, most of which are synthesized in the gastrointestinal tract by the microbial flora. Most of these vitamins are heat stable except vitamin B_1, which is heat labile.

Derivatives of the B complex vitamins **act as coenzymes**. Since they are not stored in the body, their toxicity, generally, is not observed. Two members of the vitamin B complex, i.e. folic acid and vitamin B_{12}, are important hemopoietic factors as they help in prevention of anemia.

Various members of vitamin B complex include **thiamin (B_1)**, **riboflavin (B_2)**, **niacin**, **pyridoxine (B_6)**, **pantothenic acid**, **biotin**, **choline**, **lipoic acid**, **folic acid** and **cyanocobalamin (B_{12})**.

VITAMIN B_1

Thiamin is the first member of the vitamin B complex; therefore, it is also known as **vitamin B_1**. Due to its role in prevention of beriberi, thiamin is also called **anti-beriberi factor**. Since thiamin has an important role in nervous system, it is also called **aneurine**.

Structurally, thiamin consists of a **pyrimidine ring** which is **linked to** a **thiazole ring** (Fig. 28.1).

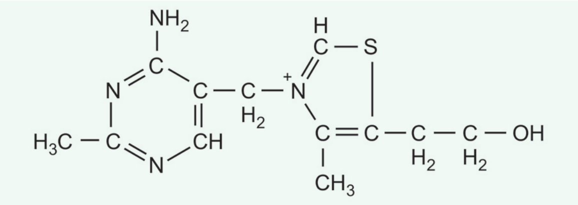


Fig. 28.1: Vitamin B_1

Thiamin occurs in nature as thiamin hydrochloride. Though thiamin is heat labile, it is quite stable in the acidic medium.

Coenzyme Form of Vitamin B_1

Vitamin B_1 (thiamin) plays an important role as a coenzyme in several biochemical reactions. **Thiamin pyrophosphate (TPP)**, i.e. the diphosphorylated form of thiamin, is referred to as coenzyme of vitamin B_1.

TPP plays an important role in carbohydrate metabolism and is used as a **coenzyme** in **oxidative decarboxylation of α-keto acids**, e.g.

$$\text{Pyruvate} \xrightarrow[\text{TPP}]{\substack{\text{Pyruvate}\\ \text{dehydrogenase}\\ \text{complex}}} \text{Acetyl CoA}$$

$$\alpha\text{-Ketoglutarate} \xrightarrow[\text{TPP}]{\substack{\alpha\text{-Ketoglutarate}\\ \text{dehydrogenase}\\ \text{complex}}} \text{Succinyl CoA}$$

TPP also acts as a coenzyme with **transketolase**, an enzyme of the hexose monophosphate shunt.

Deficiency Symptoms

Thiamin deficiency causes **beriberi**. Beriberi is of different types:

1. **Wet beriberi:** It affects cardiovascular system and results in edema, and dilated heart.
2. **Dry beriberi:** It normally affects central nervous system and causes peripheral neuropathy with myelin degeneration, leading to foot-drop, wrist-drop and sensory changes.
3. **Wernicke's encephalopathy:** It is generally observed in chronic alcoholics, who may develop CNS manifestations, ophthalmoplegia, cerebellar ataxia and mental impairment.

Thiamin deficiency may be seen in areas where people consume large quantity of the polished rice and raw fish. It is also common in chronic alcoholics.

Deficiency symptoms include nausea, vomiting, weakness and tenderness of the calf muscles.

Requirement

Average daily requirement for thiamin is **1.2–1.8 mg/day**. Its daily requirement, however, depends upon carbohydrate intake and has been defined as **0.4–0.5 mg/1000 kcal**.

Requirement is increased during fever, hyperthyroidism, and in alcoholics and pregnant women.

Sources

Rich sources of thiamin include **cereal grains**, **yeast**, liver, kidney and heart.

VITAMIN B_2

Riboflavin (**B_2**) is also called **lactoflavin**, due to its high content in milk. It is sensitive to light and ultraviolet irradiation. Riboflavin is heat stable in acidic medium.

Riboflavin has an **isoalloxazine ring** in its structure, which is attached to **ribitol** (Fig. 28.2).

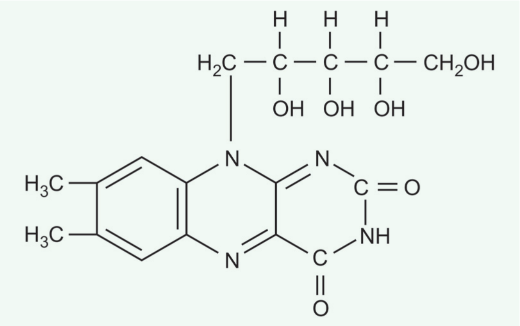


Fig. 28.2: Vitamin B_2

Coenzyme Forms of Vitamin B_2

Vitamin B_2 (riboflavin) is used as a coenzyme in various biochemical reactions. Coenzyme forms of riboflavin include FMN and FAD.

FMN (**flavin mononucleotide**) has a molecule of riboflavin and phosphoric acid. **FAD** (**flavin adenine dinucleotide**) comprises two nucleotides, i.e. FMN and AMP (Fig. 28.3).

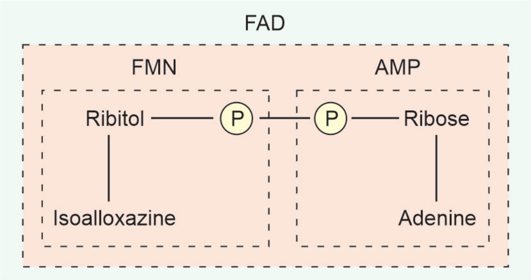


Fig. 28.3: Coenzyme forms of vitamin B_2

They form an integral part of a flavoprotein-containing enzymes and participate in several oxidation–reduction reactions, e.g.:

$$\text{FMN} \xrightarrow{2H^+} \text{FMN}\cdot H_2$$

$$\text{FAD} \xrightarrow{2H^+} \text{FAD}\cdot H_2$$

FMN is used as a coenzyme with cytochrome C reductase and **L-amino acid oxidases**.

FAD is used as a coenzyme with **succinate dehydrogenase**, **glycerol-3-phosphate dehydrogenase**, **sphingosine reductase**, **glycine oxidase**, **xanthine oxidase**, **D-amino acid oxidases** and **acyl-CoA dehydrogenase**. FAD is important in the metabolism of carbohydrates, lipids and proteins.

Deficiency Symptoms

Deficiency symptoms include **cheilosis** (lesions at angles of the mouth), **glossitis**, and ocular and skin changes such as localized dermatitis of face. There also occur functional and organic disorders of the eye, including photophobia, lacrimation, burning and itching.

Requirement

Average daily requirement of riboflavin is **1–2 mg/day** (**0.5–0.6 mg/1000 kcal**).

Sources

Milk, **liver** and kidney are rich sources of vitamin B_2.

NIACIN

Niacin is also known as **pellagra-preventive factor** (**anti-pellagra factor**) because of its role in the prevention of pellagra.

Vitamin niacin refers to two compounds, both of which are the derivatives of a **pyridine ring**. These include **nicotinic acid** and **nicotinamide** (Fig. 28.4).

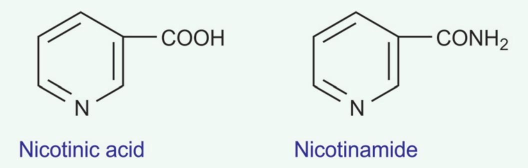
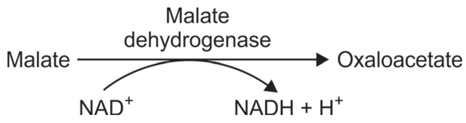

COOH
N
CONH2
N
Nicotinic acid
Nicotinamide

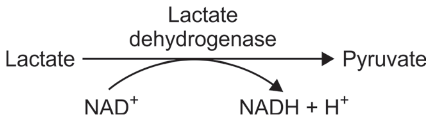
Fig. 28.4: Compounds with vitamin niacin activity

Coenzyme Forms of Niacin

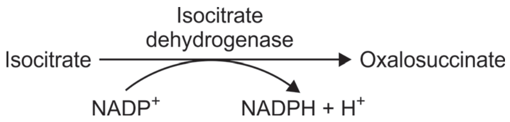
Niacin acts as a coenzyme for several biochemical reactions. There are two coenzyme forms of niacin, called NAD (**nicotinamide adenine dinucleotide**) and its phosphorylated form, referred to as NADP (**nicotinamide adenine dinucleotide phosphate**).

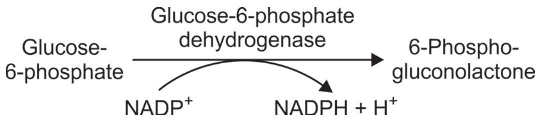
NAD^+ is used as a coenzyme with malate dehydrogenase which converts malate to oxaloacetate and lactate dehydrogenase (LDH) which converts lactate to pyruvate.


Malate dehydrogenase
Malate
Oxaloacetate
NAD+
NADH + H+

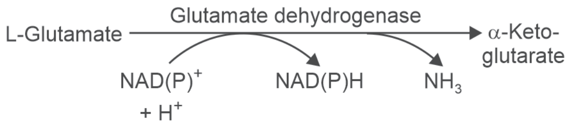

Lactate dehydrogenase
Lactate
Pyruvate
NAD+
NADH + H+

$NADP^+$ is used as a coenzyme with isocitrate dehydrogenase and glucose-6-phosphate dehydrogenase.


Isocitrate dehydrogenase
Isocitrate
Oxalosuccinate
NADP+
NADPH + H+


Glucose-6-phosphate dehydrogenase
Glucose-6-phosphate
6-Phospho-gluconolactone
NADP+
NADPH + H+

Either **NAD^+** or **$NADP^+$** can be used with glutamate dehydrogenase.


Glutamate dehydrogenase
L-Glutamate
α-Keto-glutarate
NAD(P)+ + H+
NAD(P)H
NH3

Deficiency Symptoms

Deficiency of niacin can lead to a disease called **pellagra**. It is characterized by 3Ds, i.e. dermatitis, diarrhea and dementia.

- **Dermatitis** results in pain, particularly, in those parts of the body which are exposed to sunlight such as face, neck, hands and feet. These parts become bronze, resembling sunburn, and get thickened.
- **Diarrhea** is accompanied with anorexia, abdominal discomforts and other symptoms of gastrointestinal tract.
- **Dementia** is due to impaired ability of the brain to utilize carbohydrates. Symptoms like depression, confusion and psychosis are commonly observed.

Prolonged deficiency of niacin may result in **4th D**, i.e. **death**.

Niacin deficiency is common in persons whose staple food is **maize** and **jowar**. Such individuals develop pellagra like symptoms since they are **rich in isoleucine** but **deficient in tryptophan**. This, in turn, results in amino acid imbalance, which **reduces biosynthesis of niacin** in the body.

Requirement

Average daily requirement of niacin is **12–20 mg** (**5.5–6.5 mg/1000 kcal**).

Sources

Rice polish, **yeast**, liver, poultry and green vegetables are good sources of niacin.

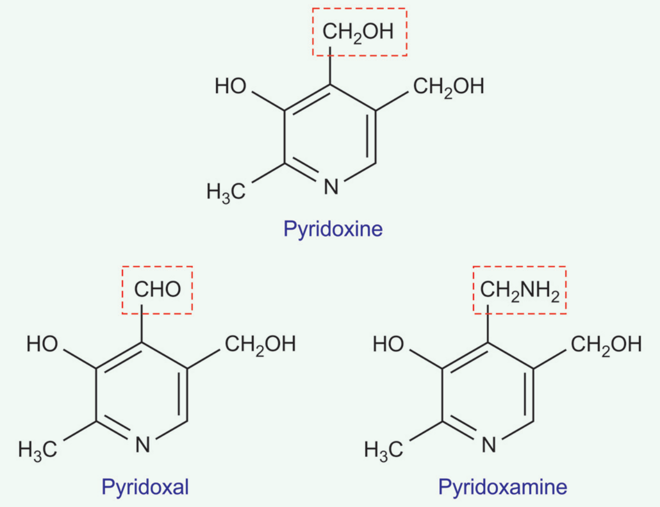
Milk and milk products, though are not an important source of niacin, but have high tryptophan content and, thus, play an important role in the prevention of pellagra.

This is due to the reason that niacin can be synthesized in the liver from tryptophan. Sixty mg of tryptophan gives one mg equivalent of niacin. This is called tryptophan–niacin conversion ratio and is 60:1.

VITAMIN B_6

Three compounds, **called pyridoxine**, **pyridoxal** and **pyridoxamine, are known to have vitamin B_6** activity.

They contain a pyridine ring and are called vitamers of vitamin B_6 (Fig. 28.5).



Pyridoxine

Pyridoxal

Pyridoxamine

Fig. 28.5: Compounds with vitamin B_6 activity

Coenzyme Form of Vitamin B_6

Vitamin B_6 acts as a coenzyme in several biochemical reactions. Coenzyme form of vitamin B_6 is referred to as **pyridoxal-5-phosphate or PLP** (Fig. 28.6).

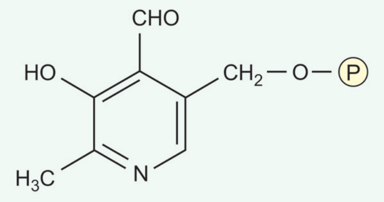


Fig. 28.6: Pyridoxal-5-P

Pyridoxal-5-phosphate performs several biochemical functions:

1. Pyridoxal-5-phosphate is a **coenzyme for glutamate pyruvate transaminase** (GPT, also called alanine aminotransferase or ALT) and **glutamate oxaloacetate transaminase** (GOT, also called aspartate aminotransferase or AST). In these reactions, PLP acts as an acceptor of the amino group from an amino acid, thereby forming the corresponding α-keto acid and pyridoxamine phosphate. Followed by the reversal of this process, pyridoxamine releases amino group to an α-keto acid and forms the corresponding amino acid (Fig. 28.7).
2. Pyridoxal-5-phosphate is also used with **dehydratases** and **desulfhydrases** required in the metabolism of hydroxy amino acids and sulfur-containing amino acids.

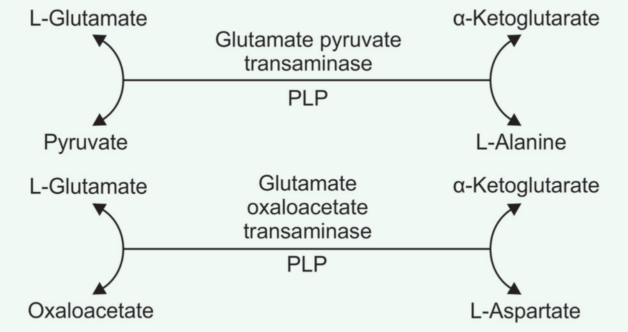


Fig. 28.7: Role of vitamin B_6 (PLP) in transamination reactions

3. Pyridoxal-5-phosphate is also used as a coenzyme with **decarboxylases**, e.g. glutamate decarboxylase, which forms γ-aminobutyric acid from glutamic acid; dopa decarboxylase that forms dopamine from dopa; and 5-hydroxytryptophan decarboxylase which forms serotonin from 5-hydroxytryptophan. Thus, vitamin B_6 also plays a role in the **regulation of neuronal activity**.
4. Pyridoxal-5-phosphate is also required by the enzyme kynureninase and plays an important role **in the conversion of tryptophan to niacin**.
5. Pyridoxal-5-phosphate is also required in the **synthesis of heme**, as it is used in the formation of δ-aminolevulinic acid, by the enzyme **δ-aminolevulinic acid synthase**.
6. Pyridoxal-5-phosphate is also essential in glycogen metabolism, both in muscle and liver, since it is also required as a coenzyme with **phosphorylase**.
7. Pyridoxal-5-phosphate is also essential for **chain elongation of unsaturated fatty acids**, e.g. in the conversion of linoleic acid to arachidonic acid.
8. Vitamin B_6 is also required for the **absorption of amino acids**.

Deficiency Symptoms

Deficiency symptoms include **cheilosis**, **glossitis** and **hypochromic anemia**.

In infants, deficiency of B_6 affects central nervous system and results in convulsions, demyelination of the peripheral nerves and degeneration of axons.

Pyridoxine has also been shown to be helpful in the treatment of nausea and vomiting during pregnancy, radiation therapy and muscular dystrophy.

Deficiency of vitamin B_6 has been shown to occur in patients with tuberculosis who are taking antitubercular drug INH (isonicotinhydrazide) since INH acts as an antagonist to vitamin B_6 (Fig. 28.8).

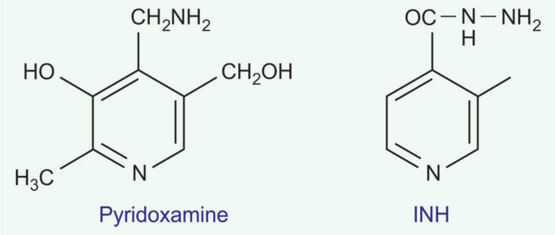


Fig. 28.8: Pyridoxamine and INH

Requirement

Daily requirement of vitamin B_6 is approximately **1–2 mg**.

Sources

Rich sources of vitamin B_6 are **egg**, **fish**, **green-leafy vegetables** and **cereals**.

PANTOTHENIC ACID

Pantothenic acid is heat stable in the acidic medium. It has a molecule of **pantoic acid** which is **linked to β-alanine** (Fig. 28.9)

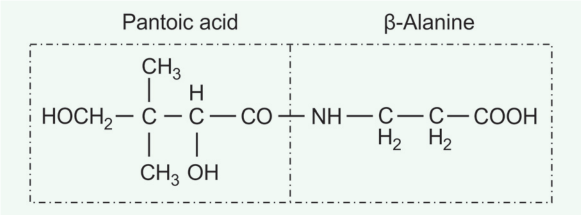


Fig. 28.9: Vitamin pantothenic acid

Coenzyme form of Pantothenic Acid

Pantothenic acid is an important constituent of coenzyme A (Fig. 28.10).

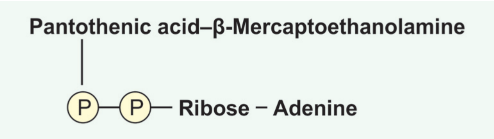


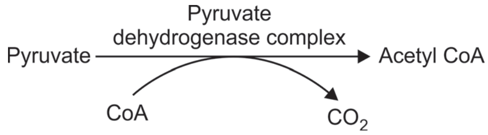
Fig. 28.10: Coenzyme A

Coenzyme A also contains adenine and a sulfhydryl group (–SH), which is contributed by β-mercaptoethanolamine.

Due to presence of the –SH group, coenzyme A is also designated as HS-CoA (CoA).

Pantothenic acid performs several functions:

1. Pantothenic acid, as 4-phosphopantotheine, forms prosthetic group of the **acyl carrier protein** which participates in **fatty acid synthesis**.
2. As a constituent of CoA, it also acts as a **carrier for the acetyl group** (as **acetyl CoA**, also called active acetate) and is used in the **biosynthesis of acetylcholine** (a transmitter at the nerve synapse), **cholesterol**, etc.
3. CoA is also used for oxidative decarboxylation of pyruvate which is converted to acetyl CoA and enters Krebs cycle.



4. **CoA** is also essential in the **metabolism of branched-chain amino acids**.
5. CoA is also required for the **activation** and **oxidation of fatty acids**.
6. CoA is also required for the **activation of succinate** and thus, plays an important role in the biosynthesis of heme.

Deficiency Symptoms

Pantothenic acid **deficiency**, in human beings, **affects nervous** and **digestive systems**. Its deficiency has also been shown to result in adrenal insufficiency.

Deficiency symptoms include headache, fatigue, impaired motor activity, muscle cramps and gastrointestinal disturbances.

Pantothenic acid is also **used in burning-feet syndrome** as well as **healing of ulcers** and **bedsores**.

Requirement

Average daily requirement of pantothenic acid is **5–10 mg**.

Sources

Rich sources of pantothenic acid are **yeast**, **liver**, **egg**, **potato**, **whole cereals** and **legumes**.

BIOTIN

Biotin is also called **anti-egg white injury factor** since it protects animals against toxicity of raw egg white.

Structurally, biotin is a **cyclic derivative of urea** (ureido ring), which is fused to a **thiophene ring** (Fig. 28.11).

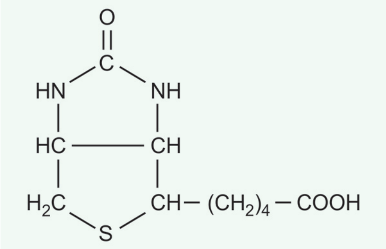
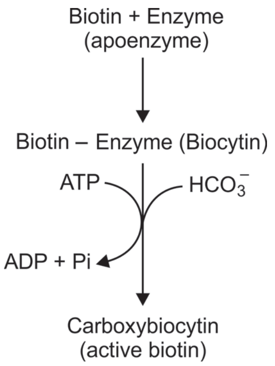


Fig. 28.11: Vitamin biotin

Coenzyme Form of Biotin

Biotin binds by the amide linkage to **ε-amino group of the lysyl residues** of the **apoenzyme** and forms holoenzyme referred to as **biocytin** (biotin-containing enzyme).


Biotin + Enzyme
(apoenzyme)
Biotin – Enzyme (Biocytin)
ATP
HCO_3^-
ADP + Pi
Carboxybiocytin
(active biotin)

Biocytin accepts CO_2 as HCO_3^- and forms a complex called **active biotin** (carboxybiocytin-enzyme complex).

Biotin is required as a coenzyme in several reactions, involving **CO_2 fixation** which are catalyzed by various carboxylases, such as:

1. Carboxylation of **pyruvate to oxaloacetate** by **pyruvate carboxylase**.
2. Conversion of propionyl CoA to **succinyl CoA** (via methylmalonyl CoA), by **propionyl-CoA carboxylase**.
3. Conversion of **acetyl CoA** to **malonyl CoA**, used in the formation of long chain fatty acids, by acetyl-CoA carboxylase.

Deficiency Symptoms

Deficiency of biotin may cause **mild dermatitis**, **nausea and loss of appetite**, **muscular pain** and **anemia**.

Biotin deficiency is not generally seen, as intestinal flora can synthesize it.

Biotin deficiency is induced by taking large amount of raw egg white, which contains a heat labile, biotin binding, protein, called avidin. Avidin binds to biotin and inhibits its absorption. Heating egg white prevents it, since heating denatures avidin.

Requirement

Daily requirement of biotin is **15–30 µg**.

Sources

Rice polish, **whole cereals**, **milk**, **yeast** and **organ meat** such as **liver** and **kidney** are good sources of biotin.

FOLIC ACID

Folic acid and the related compounds are referred to as **folacin**, since it is derived from the word folium (means leaf).

Folic acid is also called **pteroylglutamic acid**. It has a **pteridine nucleus**, a molecule of **p-aminobenzoic acid** and several **glutamate molecules as a part of its structure**. Number of glutamate molecules may vary from one to seven (Fig. 28.12).

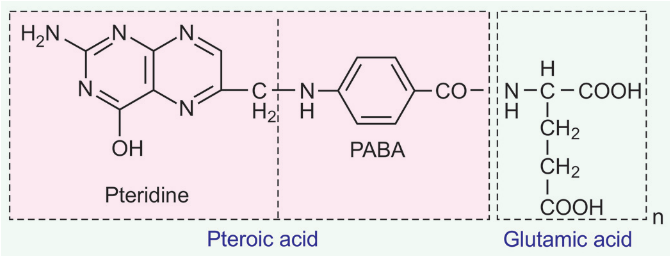

H_2N
N
N
N
N
OH
Pteridine
C
H_2
N
H
PABA
CO
N
H
H
C
COOH
CH_2
CH_2
COOH
n
Pteroic acid
Glutamic acid

Fig. 28.12: Vitamin folic acid

Coenzyme form of Folic Acid

Coenzyme form of folic acid is referred to as **tetrahydrofolate (FH_4)**. **During its synthesis**, **folate** is firstly **reduced** by **folate reductase to dihydrofolate** (FH_2) and, subsequently, by **dihydrofolate reductase** to **FH_4** (Fig. 28.13).

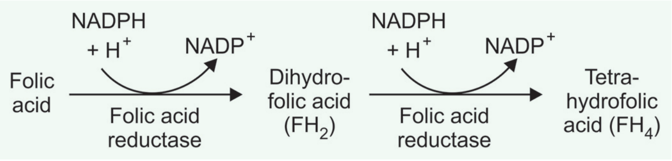

Folic acid
NADPH
+ H^+
$NADP^+$
Folic acid reductase
Dihydro-folic acid (FH_2)
NADPH
+ H^+
$NADP^+$
Folic acid reductase
Tetra-hydrofolic acid (FH_4)

Fig. 28.13: Conversion of folic acid to FH_4 (coenzyme)

Tetrahydrofolate (FH_4) acts as an **acceptor** as well as **donor** of **one-carbon unit** (1-C) in several reactions, in the metabolism of amino acids and nucleotides.

Sources of **one-carbon moiety include histidine** (formimino group), **serine** (hydroxymethyl group), and **choline** and **methionine** (methyl group).

One-carbon moiety carried by FH_4 is incorporated as carbon-2 and C-8 in the biosynthesis of a purine. It is also used in the conversion of **glycine to serine**, **uracil to thymine** and **homocysteine to methionine**. It is also used in the **biosynthesis of choline**.

Some of these reactions also require vitamin B_{12}.

Deficiency Symptoms

Folic acid deficiency may result in **macrocytic** (**megaloblastic**) anemia.

Other deficiency symptoms such as **psychosis** and mental retardation are due to combined deficiency of folic acid and vitamin B_{12}.

Folic acid deficiency (in early pregnancy) has also been implicated in **neural tube defects**.

Folic acid deficiency may be seen in stressful conditions, during pregnancy, intestinal malabsorption such as steatorrhea and during antibiotic therapy.

Requirement

Average daily requirement of folic acid, for an adult, is **150–300 μg**.

Sources

Green leafy vegetables such as **spinach** and **cabbage**, **yeast**, **liver** and **kidney** are good sources of folic acid.

VITAMIN B_{12}

Vitamin B_{12} is also called **extrinsic factor of Castle**. Due to presence of the **cyano** group and **cobalt**, vitamin B_{12} is also referred to as **cyanocobalamin**.

Ring structure of the vitamin has a **corrin nucleus**. Cobalt is linked to the cyano group as well as to nitrogen of the imidazole ring, which is esterified through aminopropanol and propionic acid to ring IV of the corrin nucleus (Fig. 28.14).

Coenzyme Form of Vitamin B_{12}

Vitamin B_{12} is used as a coenzyme in a number of reactions. There are two **coenzyme forms** of the vitamin. These are called **methylcobalamin** and **deoxyadenosylcobalamin**. These are referred to as **cobamides** (cobamide coenzymes).

Methylcobalamin is used as a **coenzyme** in the **formation of methionine** from **homocysteine**. Thus, vitamin B_{12} helps in maintaining methionine stores of the body and spares FH_4 for use in the synthesis of purines and pyrimidines.

Deoxyadenosylcobalamin is important in the metabolism of branched-chain fatty acids as well as fatty acids containing odd number of carbon atoms, by converting methylmalonyl CoA to succinyl CoA by **methylmalonyl CoA isomerase**.

Deficiency Symptoms

Vitamin B_{12} deficiency can result in **methylmalonic aciduria**. Measurement of the urinary excretion of methylmalonic acid is used to confirm deficiency of this vitamin.

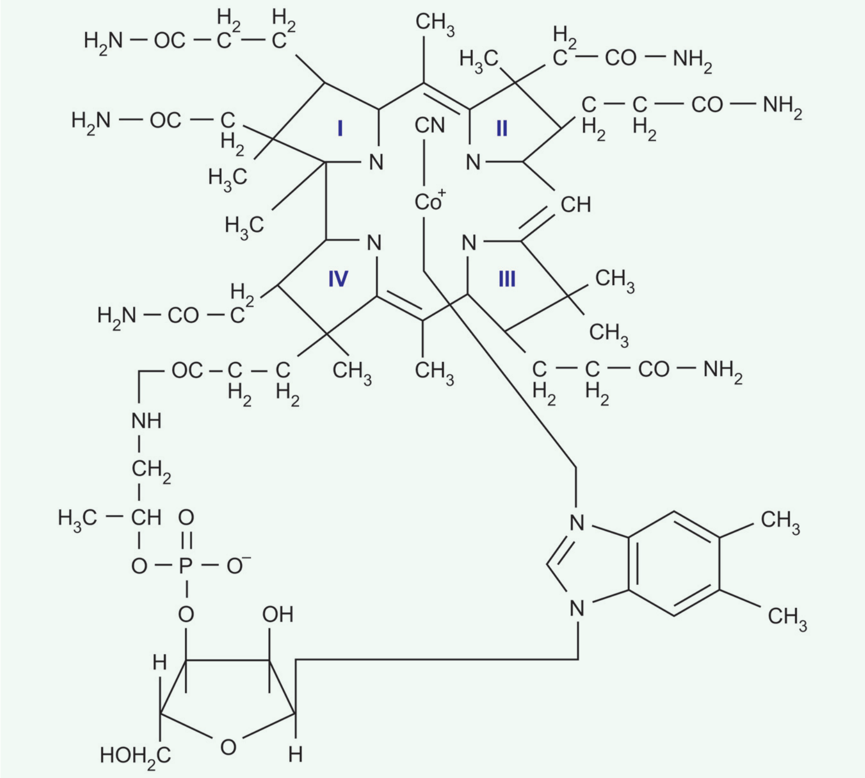


Fig. 28.14: Vitamin B_{12}

Vitamin B_{12} deficiency is not generally observed due to its widespread nature in animal tissues as well as its synthesis by microbial flora. Deficiency, however, can occur in geriatric persons, particularly, in those who are strict vegetarians.

Its deficiency may also occur on its prolonged reduced intake, intestinal malabsorption, atrophy of the gastric mucosa, or due to the **deficiency of the intrinsic factor**, e.g. in pernicious anemia.

Requirement

Daily requirement of vitamin B_{12} is **1–3 µg**.

Sources

Liver, **kidney**, **egg**, **fish** and **milk** are the good sources of vitamin B_{12}.

VITAMIN C

Vitamin C is also called **ascorbic acid**, **antiscorbutic factor**, or **antiscurvy factor**.

Majority of the animals can synthesize vitamin C from glucuronate but men, primates and guinea pigs cannot synthesize it.

Vitamin C is readily absorbed from the intestinal tract. Its absorption is reduced during gastrointestinal infections and in achlorhydria.

Though it is stored in the liver and the adrenals, highest concentration is found in the adrenal cortex.

Vitamin C is destroyed by heat.

Biochemical Functions of Vitamin C

Vitamin C has an en-diol group at the second and the third carbons due to which it is sensitive to oxidizing agents. Oxidation of ascorbic acid converts it to **dehydroascorbic acid** (Fig. 28.15)

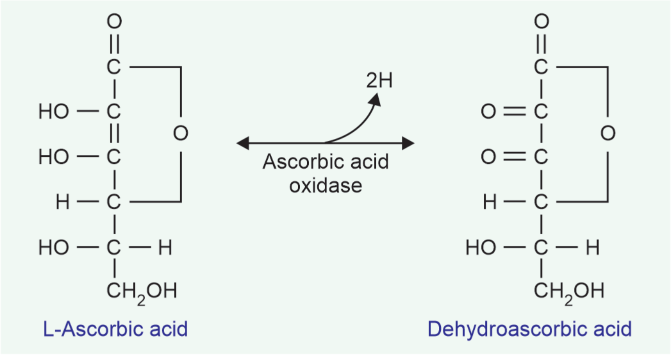


Fig. 28.15: Reduced and oxidized forms of vitamin C (ascorbic acid)

Due to its oxidation–reduction properties, ascorbic acid performs several biological functions in the body:

1. Ascorbic acid is required in the **hydroxylation of amino acids**, particularly, the aromatic amino acids.
2. Due to its role in the **hydroxylation of proline** and **lysine** (**in collagen**), ascorbic acid is required for the **repair of the ground substance**.
3. Vitamin C is essential for the **absorption of iron** from the gastrointestinal tract as well as its **release from ferritin**.
4. Vitamin C has **stimulating** effect on **phagocytic activity** of leucocytes. It, thus, provides resistance to the body.
5. **Ascorbic acid** is also required for the **synthesis of the steroid hormones** in the adrenal cortex. It, thus, helps in lowering blood cholesterol level.

Deficiency Symptoms

Prolonged deficiency of vitamin C causes **scurvy** which is characterized by **multiple hemorrhages**. Early symptoms of the disease include **swollen**, **spongy and bleeding gums**; **loosening of teeth**; and **pain in joints**. In severe cases, there may also be **bleeding from nose**, **gastrointestinal tract** and **genitourinary tract**.

Infants may **lose appetite and body weight** with **painful tenderness of the extremities**, bleeding from **gums** and **mucous membrane**, and **cessation of osteogenesis** of long bones.

Because of its role in absorption, transport and storage of iron, vitamin C deficiency may cause **anemia**.

Requirement

Daily requirement of vitamin C for adults is **40–60 mg**.

Sources

Amla is the **richest source of vitamin C**. **Citrus fruits**, **green chilies**, **guava** and **tomatoes** are also good sources of vitamin C.

Hypervitaminosis C

Vitamin C may be toxic, if taken in large doses. More than 2 g, in a single dose, may result in abdominal pain, diarrhea, and nausea. More than 3 g/day may **lead to urolithiasis** due to the formation of **oxalate stones**.

Various water soluble vitamins, their coenzyme forms, major biochemical functions, principal deficiency diseases and daily requirements are shown in Table 28.1

FAT SOLUBLE VITAMINS

Fat soluble vitamins are vitamins **A**, **D**, **E** and **K**. They are absorbed in the lumen, in the presence of fat and are emulsified with bile.

Their **precursors** are called **provitamins**, which mostly occur in plants.

Due to fat soluble nature of these vitamins, they are **stored in the liver** and several other tissues. **Large doses of these vitamins** particularly, for a fairly long duration, may result in **hypervitaminosis** leading to various toxic symptoms.

VITAMIN A

Vitamin A is also called **anti-night blindness factor**. It has a characteristic 6-membered ring, known as **β-ionine ring**, which is attached to a long **hydrocarbon side chain** (Fig. 28.16)

Vitamin A (**retinoids**) actually refers to three preformed compounds that exhibit metabolic activity, i.e. the alcohol (**retinol**), the aldehyde (retinaldehyde or **retinal**) and the acid (**retinoic acid**). Stored retinol is often esterified to a fatty acid, usually palmitate, and is called retinylpalmitate. These retinyl esters are also usually found complexed with proteins in foods. These active forms of vitamin A **exist in only animal products**.

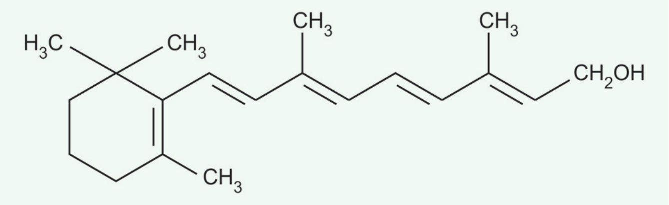


Fig. 28.16: Vitamin A

Provitamin A

Carotenoids are called **provitamin A**. They are **found in plants** and **have** an **additional ionine ring**. Depending upon the presence of the second ionine ring, provitamins are designated as **α**, **β** and **γ-carotenes**.

β-Carotene has **highest vitamin A activity**, as it gives **2 molecules of vitamin A** (retinal). On the other hand, **α-** and **γ-carotenes** give **only one** molecule. **Provitamins** (carotenoids) are absorbed in the lumen and **converted to retinal in the intestinal epithelium**. Carotenes are splitted at the center of the hydrocarbon chain and give rise to active vitamin A within the intestinal wall. β-Carotene is found widely in plants. Its rich **sources** are **yellow-colored fruits** and **vegetables** such as **carrots**, **pumpkin**, **papaya** and **mango**.

Table 28.1 Water soluble vitamins, their coenzyme forms, biochemical role, deficiency diseases and daily requirement for adults

Vitamin	Coenzyme	Major biochemical roles	Deficiency diseases	Daily requirements
Vitamin B complex				
Thiamin (B_1)	TPP	Oxidative decarboxylation	Beriberi	1.2–1.8 mg
Riboflavin (B_2)	FMN, FAD	Oxidation-reduction with flavoproteins	Lesions on lips, cheilosis	1–2 mg
Niacin	NAD^+, $NADP^+$	Hydrogen acceptor for dehydrogenases	Pellagra	12–20 mg
Pyridoxine (B_6)	Pyridoxal-5-PO_4	Transamination, decarboxylation	Peripheral neuritis	1–2 mg
Pantothenic acid	Coenzyme A	Formation of CoA, component of the acyl carrier protein	Muscle cramps, burning feet	5–10 mg
Biotin	Carboxybiocytin	Cocarboxylase	Anemia, dermatitis	15–30 µg
Folic acid	FH_4	Carrier of one-C moiety	Megaloblastic macrocytic anemia	150–300 µg
Cobalamin (B_{12})	Cobamides	Transmethylation with FH_4	Pernicious anemia	1–3 µg
Vitamin C (Ascorbic acid)	L-Ascorbate	Hydroxylation	Scurvy	40–60 mg

Conversion of β-carotene to retinal requires an enzyme referred to as dioxygenase (Fig. 28.17).

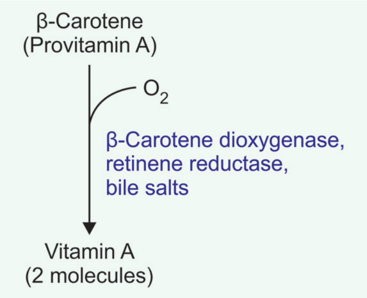

β-Carotene
(Provitamin A)
O_2
β-Carotene dioxygenase,
retinene reductase,
bile salts
Vitamin A
(2 molecules)

Fig. 28.17: Conversion of β-carotene to vitamin A

Physiological Functions of Vitamin A

1. **Role in visual process: Vitamin A aldehyde (retinal) is essential for vision, both in rods as well as cones:**
   - **In rods:** Rod cells contain a conjugated **transmembrane protein** called **rhodopsin**, which consists of an apoprotein **opsin** and a nonprotein prosthetic group called **11-cis-retinal** (11-cis-retinene). It is **essential for vision in dim light**.
     When light falls on retina, **rhodopsin** is **bleached** and generates **all-trans-retinal**, which cannot form a complex with opsin. **All-trans-retinal** is **reduced to all-trans-retinol** by the enzyme all-trans-retinol dehydrogenase (retinene reductase). It is then transported to liver and **stored there**. In the liver, it is **isomerized** to **11-cis-retinol** by an isomerase. After its delivery to the retina, **11-cis-retinol** is **changed to 11-cis-retinal**, which again conjugates **with opsin** and reforms **rhodopsin**, **in dark**. This is called vision cycle (Fig. 28.18).

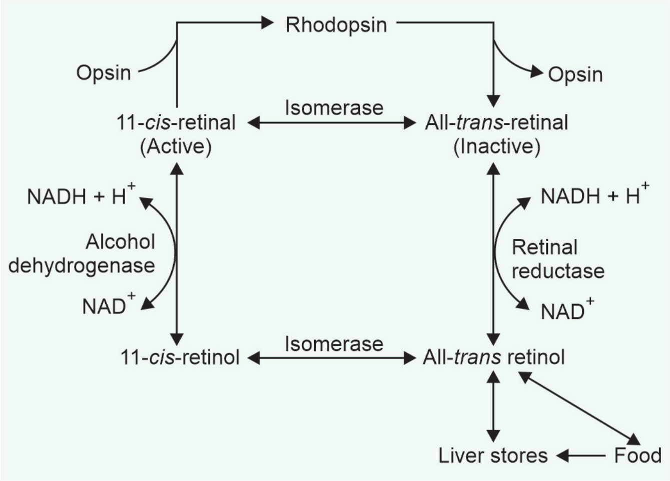

Rhodopsin
Opsin
Opsin
Isomerase
11-*cis*-retinal
(Active)
All-*trans*-retinal
(Inactive)
NADH + H⁺
Alcohol
dehydrogenase
NAD⁺
NADH + H⁺
Retinal
reductase
NAD⁺
Isomerase
11-*cis*-retinol
All-*trans* retinol
Liver stores
Food

Fig. 28.18: Role of vitamin A (retinal) in vision cycle

   - **In cones:** Similar to rhodopsin, which is present in rods for the black and white vision, cones contain 3 different pigments that are sensitive to three primary colors, i.e. **porphyropsin** (**for red**), **iodopsin** (**for green**) and **cyanopsin** (**for blue**). Prosthetic group in these pigments is the same, i.e. 11-*cis*-retinal.
2. Vitamin A deficiency leads to reduction **in gluconeogenesis**. This is due to decreased **synthesis of steroid hormones** particularly, synthesis of corticosterone and cortisol.
3. Vitamin A also plays a role in cholesterol biosynthesis.
4. Vitamin A deficiency causes keratinization of the mucosal membrane and **decreases synthesis of glycosaminoglycans**.
5. Vitamin A deficiency **impairs spermatogenesis and affects cell growth and differentiation**.
6. Deficiency of vitamin A also **reduces synthesis** of membrane **glycoproteins**.
7. Vitamin A is also required in the maintenance of integrity of the tissue lining eyes, gastrointestinal tract, genitourinary tract and respiratory system, and ensures **optimal functioning of the immune system**.

Deficiency Symptoms

Vitamin A deficiency is observed, primarily due to its low intake. Its deficiency is aggravated by infections, which reduces its absorption and utilization. Vitamin A deficiency affects many tissues:

1. It is most detrimental for eyes. Deficiency of the vitamin may result in **xerophthalmia**, which is characterized by drying of conjunctiva and cornea, followed by destruction of cornea, leading to blindness. Manifestations of xerophthalmia are shown in Fig. 28.19.

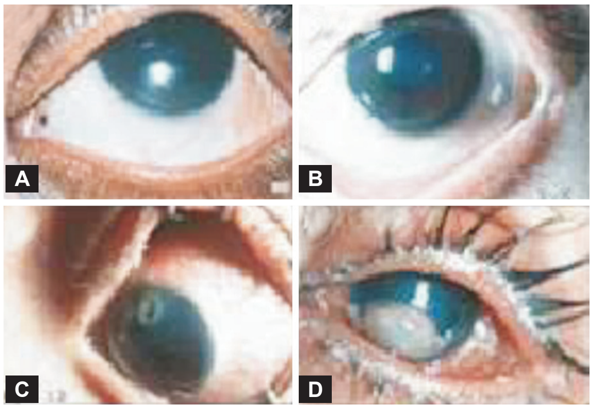


Fig. 28.19: Manifestations of xerophthalmia. (A) Bitot's spot; (B) Corneal xerosis; (C) Keratomalacia; and (D) Corneal scar

2. Vitamin A deficiency also affects **osteoblastic activity** and results in **defective resorption of bone**, affecting **growth**. Deficiency of vitamin A also results in **xerosis** and **keratinization of the mucous membrane**, resulting in **dry** and **rough skin**. Keratinization of the mucous membrane increases its susceptibility to infections. Keratinization of the urinary tract may lead to **urolithiasis**.
3. Vitamin A deficiency also results in certain **abnormalities** of the **reproductive** system, such as **degeneration of testes**, **abortions** and production of the **malformed-offspring**. Keratinization of epithelium of the seminiferous tubules may also lead to infertility.

Requirement

Average daily requirement of **vitamin A** for an adult is **2000–3500 IU** (about 750–1000 µg).

Daily requirement of **β-carotene** is **4000–6000 IU** (about 2000 µg).

Sources

Liver oil, **fish-liver oil**, **egg**, **milk** and milk products are the good sources of **vitamin A**.

On the other hand, **papaya**, **carrots**, sweet potato, and green leafy vegetables are the good sources of β-carotene.

Hypervitaminosis A

If taken in large quantities for a longer period, vitamin A is toxic since it is a fat soluble vitamin and stored in the liver.

Toxicity symptoms include **skin abnormalities** such as **dry and rough skin**, **scaly dermatitis**, headache, nausea, vomiting and loss of appetite, **hepatomegaly**, painful joints and **several bone abnormalities**.

VITAMIN D

Vitamin D (**calciferol**) is a sterol. It is also called **anti-rachitic factor** and is considered to be a prohormone. It is present in nature in several forms, two of which are most common. These are referred to as vitamins D_2 and D_3. **Vitamin D_2** (**ergocalciferol**) is of **plant origin** and can be obtained from ergot plant. It can also be synthesized in the laboratory by irradiating ergosterol. **Vitamin D_3** (**cholecalciferol**) is of animal origin (Fig. 28.20).

Provitamin D

A derivative of the cholesterol, called 7-dehydrocholesterol, is a precursor of vitamin D_3 in animals. It is also called **provitamin D**. It is synthesized in the human beings and is converted to vitamin D_3 by **UV light, in the skin**. This process is referred to as **photobiogenesis**.

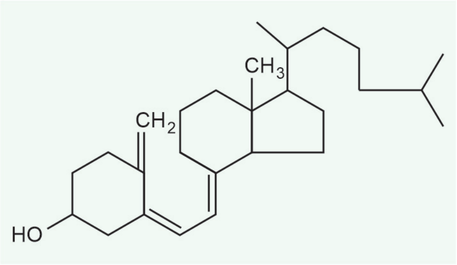


Fig. 28.20: Vitamin D_3 (cholecalciferol)

Active Vitamin D

Vitamin D is activated **by two sequential hydroxylations**. The first step takes place in the **liver** where it is hydroxylated by cytochrome P_{450}-dependent **25-hydroxylase** to **25-hydroxycholecalciferol [25(OH).D_3]**.

From liver, 25-hydroxycholecalciferol is transported to **kidney** where it is further hydroxylated at position-1 by the mitochondrial enzyme **1-α-hydroxylase**, forming **1,25-dihydroxycholecalciferol [1,25$(OH)_2$.D_3]**. This is the physiologically **active** form of the vitamin which functions as a **hormone** and is essential for calcium homeostasis (Fig. 28.21).

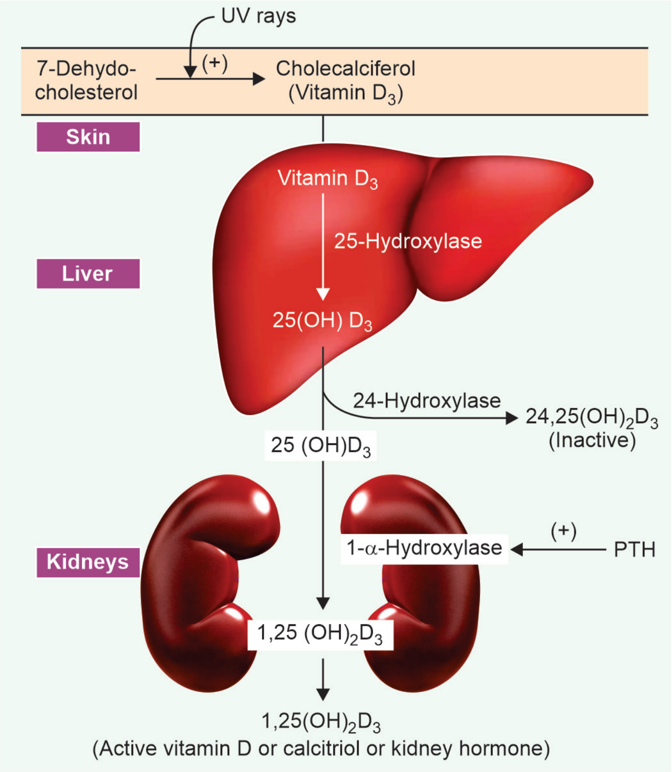


Fig. 28.21: Synthesis of active vitamin D_3 (calcitriol)

Due to presence of the 3-OH groups, 1, 25$(OH)_2$. D_3 is also called **calcitriol**.

1-α-Hydroxylase activity is present in the renal tubules, bone and placenta. Parathyroid hormone (PTH), serum phosphorus and calcitriol regulate its activity. Production of **1,25$(OH)_2$.D_3** is also **regulated by serum calcium**.

Physiological Functions of Vitamin D

Most important function of active vitamin D is to regulate serum calcium level.

Role of vitamin D in Calcium Metabolism

Calcitriol induces synthesis of calcium-binding protein, Ca^{2+}-dependent ATPase and alkaline phosphatase, which are essential for the regulation of serum calcium and phosphorus.

Net result is achieved through its action in the intestine, bone and kidney:

- **In the intestine**, due to **stimulation of the synthesis** of the **calcium-binding protein**, 1,25$(OH)_2$.D_3 increases intestinal absorption of calcium as well as phosphorus.
- **In the bone**, calcitriol **stimulates synthesis of osteocalcin**, which, in turn, mobilizes calcium from bone, i.e. it causes **demineralization of bone**, and
- **In the kidney**, active vitamin D **increases tubular reabsorption of calcium** and excretion of phosphorus.

Overall effect of the three processes, thus, is to **increase serum calcium** level towards normal.

Deficiency Symptoms

Deficiency of vitamin D, generally, does not occur, as it can be synthesized in the body. Deficiency however, may occur due to lack of sunshine.

Vitamin D deficiency leads to **rickets** in **children** and **osteomalacia** in **adults** (Fig. 28.22).

Requirement

Average daily requirement is **200 IU** (5–10 μg) of cholecalciferol.

Sources

As vitamin D can be synthesized in the body by the action of the ultraviolet light on the skin, hence, sunshine is the best source of vitamin D. Besides sunshine, rich sources of vitamin D are **milk**, **butter**, **egg yolk**, **fish oil** and **liver oil**.

Hypervitaminosis D

Extremely **large doses** of vitamin D, over a long period, may lead to **hypercalcemia**, **hyperphosphatemia**, **anorexia**, **nausea**, **vomiting** and **diarrhea** besides bone pain and **metastatic calcification of the soft tissues** including kidney, myocardium, pancreas and uterus.

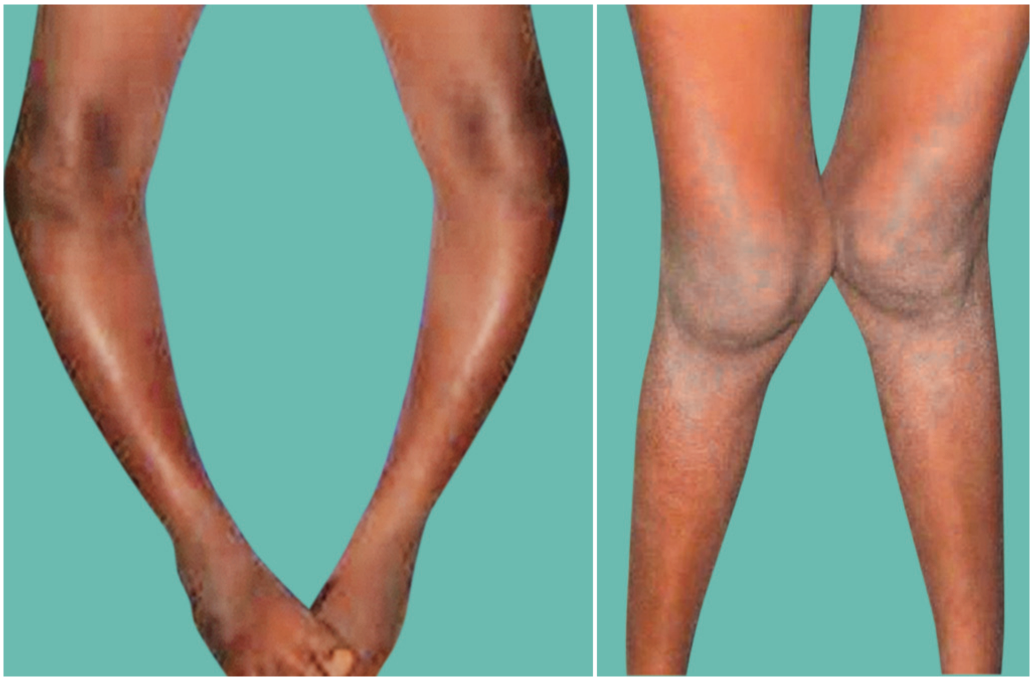


Fig. 28.22: Rickets (bowed-legs and knock-knees)

VITAMIN E

Several compounds belonging to the group of tocopherols, such as α, β, γ and δ tocopherols, exhibit vitamin E activity. Amongst them, α-tocopherol has highest biological activity as well as widest distribution. It is an alcohol with a **phytol** and **trimethylhydroquinone** (Fig. 28.23).

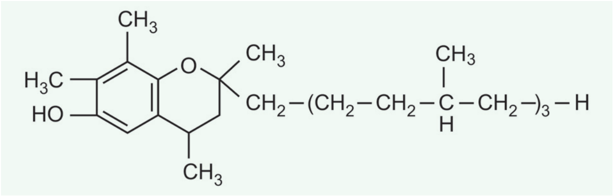


Fig. 28.23: Vitamin E

Commercially, vitamin E is available both as a natural substance as well as a synthetic compound. **Natural** vitamin E is **d-α-tocopherol** and is a single stereoisomer. On the other hand, **synthetic** vitamin E is **dl-α-tocopherol**, which is produced commercially.

Physiological Functions of Vitamin E

Most important function of vitamin E is as an **antioxidant** since it has the ability to **quench free radicals**, which are generated by metabolic processes and environmental pollutants.

Deficiency Symptoms

Vitamin E deficiency **results in** a range of **degenerative diseases**.

Its deficiency is seen **in patients with abetalipoproteinemia**, who cannot form chylomicrons and VLDL. As a result of **abetalipoproteinemia**, vitamin E can neither be absorbed nor distributed.

Deficiency of vitamin E is also seen in children with fat malabsorption, where it results in severe progressive **neuropathy** and **retinopathy**.

Hepatic necrosis, muscular dystrophy, defects in vascular system and certain reproductive defects are also known to occur due to deficiency of vitamin E in various animal species.

Requirement

Daily requirement of vitamin E for an adult man is 5–10 mg (about **15 IU**).

Requirement of vitamin E varies depending upon the level and nature of physical activity, diet, psychological stress, exposure to pollutants and intake of polyunsaturated fatty acids. Normally, 0.4 mg of vitamin E is required per g of PUFAs.

Sources

Tocopherols are present in small quantities **in green leafy vegetables** such as spinach and lettuce, milk and milk products, and **egg yolk**. Oils, such as **corn oil**, **cottonseed oil** and **sunflower oil** are the rich sources of vitamin E.

VITAMIN K

The name **vitamin K** has been derived from the Danish word 'Koagulation' since it has an important **role in blood clotting**.

Various compounds related to **2-methyl-1,4-napthoquinone** have been shown to possess vitamin K activity.

The most important form of vitamin K is **K_1**, also called **phylloquinone**. It occurs naturally and is found from plant sources. Vitamin K_1 has phytyl side chain (Fig. 28.24).

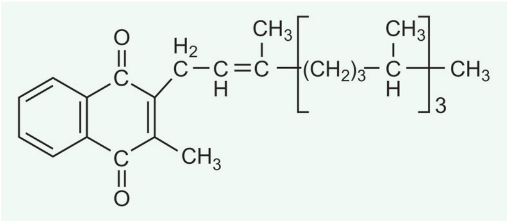


Fig. 28.24: Vitamin K

Vitamin K_2, also called **menaquinone** or **farnoquinone**, is synthesized by the intestinal bacteria. It has farnesyl side chain.

Vitamin K_3, also called **menadione**, is a synthetic compound. It is water soluble and gets easily absorbed on parenteral administration. Vitamin K which is commercially available for therapeutic use, is menadione.

Physiological Functions of Vitamin K

Vitamin K is essential for **maintenance of the normal levels** of various **blood clotting factors** such as **prothrombin**, **factor VII**, **factor IX** and **factor X**. **These proteins** are **synthesized** in the liver **in their inactive forms** and get **converted** to their **biologically active**

forms by γ-carboxylation, with the help of vitamin K (Fig. 28.25).

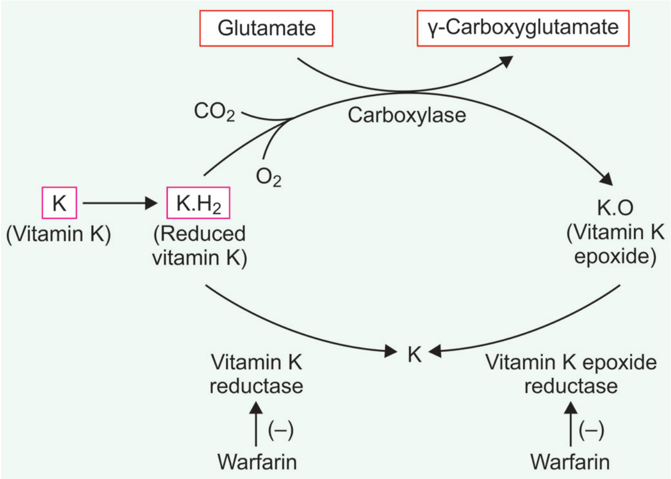


Fig. 28.25: Role of vitamin K in carboxylation of blood clotting factors

Deficiency Symptoms

Deficiency of vitamin K is seen in newborns, due to their sterile intestine, particularly in premature infants who show **reduced** levels of **vitamin K**, **prothrombin** and **Christmas factor**.

Common **deficiency symptoms** include **cutaneous** and **intramuscular hemorrhages** with **bluish-red discoloration** in different parts of the body.

Requirement

Its requirement is not well established in adults but it is suggested that **20–100 µg/day** is sufficient to meet its daily needs. It is advised to administer a **single** prophylactic **dose of 1 mg** of vitamin K_1 to a newborn on the **first day of life**.

Sources

Green leafy vegetables such as spinach, lettuce and cabbage, are the rich sources of vitamin K. **Fish, liver** and **skeletal muscle** are also good sources of this vitamin.

Vitamin K Antagonists

Anticoagulants such as **dicumarol** and **warfarin**, due to their structural similarities with vitamin $K_{1,}$ act as antagonists (anti-vitamins) of vitamin K. They act as **competitive inhibitors for the enzymes** which are concerned with the activation of prothrombin and other coagulation factors, and require vitamin K for their activation.

Table 28.2 lists various fat soluble vitamins, their biological functions, deficiency diseases, sources and daily requirements for adults.

Table 28.2 Fat soluble vitamins, their biological functions, deficiency diseases, sources and daily requirement for adults

Vitamin	Provitamin	Biological functions	Deficiency diseases	Sources	Daily requirement
Vitamin A (Retinol)	β-Carotene (Carotenoids)	In vision cycle, growth and maintenance of epithelium	Night blindness; disorders of mucous membrane, bone, nerves; and reproduction	Liver oil, fish oil and vegetable oils	2000–3500 IU
Vitamin D (Calciferol)	7-Dehydrocholesterol	Regulation of serum calcium and phosphorus	Rickets and osteomalacia	Fish oil	200 IU
Vitamin E (Tocopherols)	—	As antioxidant	Hemolytic anemia (in infants) and muscular dystrophy	Plant seed oils	5–10 mg
Vitamin K (Phylloquinone)	—	In blood coagulation	Blood clotting failure	Plant seed oils	20–100 µg

Important Questions

1. Name fat soluble vitamins? Describe their dietary sources, physiological functions and deficiency disorders.
2. Describe coenzyme forms and biochemical functions of:
    - i. Riboflavin
    - ii. Pantothenic acid
    - iii. Biotin
    - iv. Vitamin B6
    - v. Thiamin
    - vi. Niacin
3. Discuss briefly:
    - i. Functions of vitamin D
    - ii. Role of vitamin A in vision
4. Write notes on:
    - i. Pellagra
    - ii. Beriberi
    - iii. Scurvy
    - iv. Cobamide coenzymes
    - v. Calcitriol
    - vi. Role of vitamin D in calcium metabolism
    - vii. Role of vitamin K in gamma-carboxylation
    - viii. Pyridoxine
    - ix. Wernicke's encephalopathy
    - x. Provitamin A
    - xi. Xerophthalmia
    - xii. Provitamin D
    - xiii. Active vitamin D
    - xiv. Rickets
    - xv. Vitamin K antagonists
    - xvi. Hypervitaminosis

Chapter

29

Metabolism of Carbohydrates

As discussed earlier (Chapter 5), all the dietary carbohydrates are digested and absorbed as glucose, which enters the liver. In the liver, glucose is utilized in various metabolic pathways which include glycolysis (oxidized to produce energy), glycogenesis (stored as glycogen in the liver and muscle) and pentose phosphate pathway (to produce pentoses and NADPH). During starvation, when blood glucose is low, glucose is released from glycogen (glycogenolysis) or produced via gluconeogenesis (Fig. 29.1).

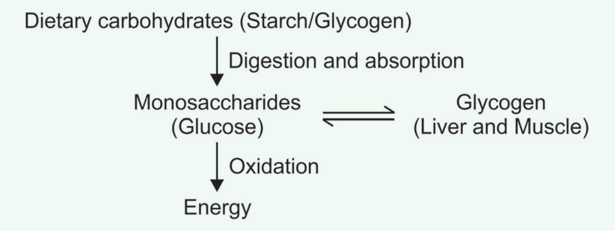

Dietary carbohydrates (Starch/Glycogen)
Digestion and absorption
Monosaccharides
(Glucose)
Glycogen
(Liver and Muscle)
Oxidation
Energy

Fig. 29.1: Metabolic fates of glucose

GLYCOLYSIS

Glycolysis, also called Embden Meyerhof (or Embden Meyerhof Parnas) pathway, is a process of utilization (catabolism) of glucose, mainly, in the liver (aerobic glycolysis) and skeletal muscle (anaerobic glycolysis). Glycolytic enzymes are present in the extramitochondrial compartment of the cell to produce energy.

Outline of Glycolysis

Various reactions of the glycolytic pathway are outlined in Fig. 29.2.

1. Glucose is first converted to glucose-6-phosphate. This reaction is catalyzed by the enzyme hexokinase, which requires ATP and Mg. Hexokinase is present in all the tissues.

   In the fed state, this reaction is catalyzed by another enzyme, called glucokinase. This enzyme is found

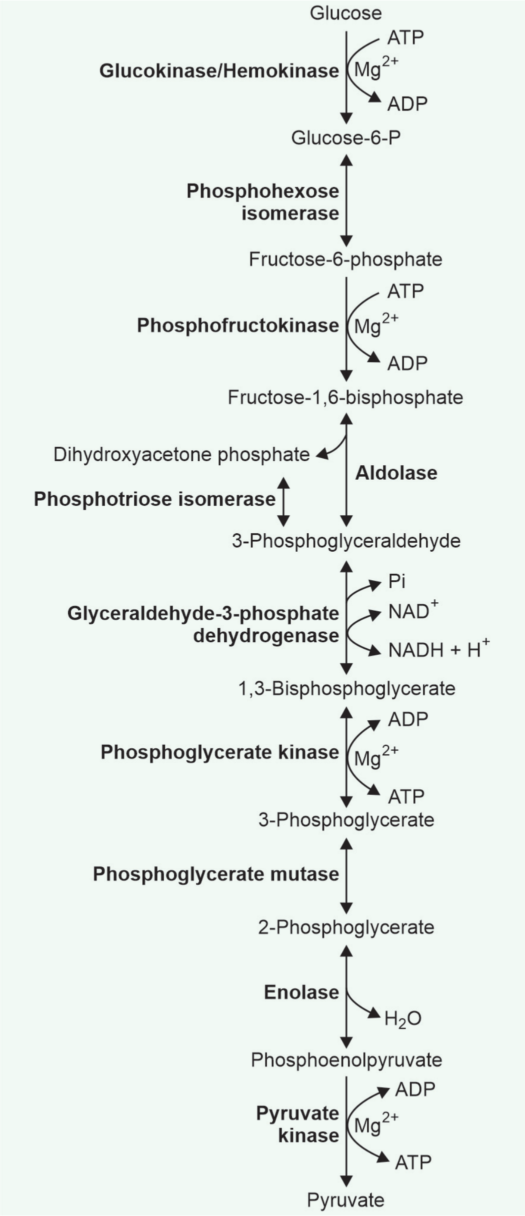
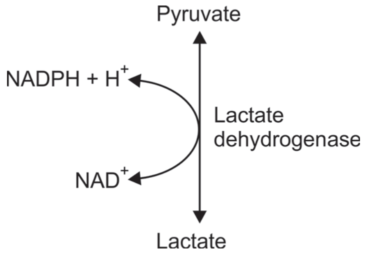

Glucose
ATP
Glucokinase/Hemokinase
Mg^{2+}
ADP
Glucose-6-P
Phosphohexose isomerase
Fructose-6-phosphate
ATP
Phosphofructokinase
Mg^{2+}
ADP
Fructose-1,6-bisphosphate
Dihydroxyacetone phosphate
Aldolase
Phosphotriose isomerase
3-Phosphoglyceraldehyde
Pi
Glyceraldehyde-3-phosphate dehydrogenase
NAD^+
$NADH + H^+$
1,3-Bisphosphoglycerate
ADP
Phosphoglycerate kinase
Mg^{2+}
ATP
3-Phosphoglycerate
Phosphoglycerate mutase
2-Phosphoglycerate
Enolase
H_2O
Phosphoenolpyruvate
ADP
Pyruvate kinase
Mg^{2+}
ATP
Pyruvate

Fig. 29.2: Outline of glycolysis

in the liver, where it is induced by high glucose concentration, found after meals. It has low affinity (high Km) for glucose and its function is to remove excess of glucose from blood, in the fed state.

2. In the next reaction, glucose-6-phosphate is isomerized, in a freely reversible reaction, to fructose-6-phosphate, by the enzyme phosphohexose isomerase.
3. Fructose-6-phosphate is then phosphorylated by an allosteric enzyme phosphofructokinase to form fructose-1,6-bisphosphate.
   This is an irreversible reaction and requires ATP. ATP acts as a co-substrate as well as allosteric inhibitor of the enzyme.
4. Thereafter, fructose-1,6-bisphosphate (a hexose) is cleaved by aldolase to 3-phosphoglyceraldehyde (glyceraldehyde-3-phosphate) and dihydroxyacetone phosphate, two trioses.
5. Subsequently, since only 3-phosphoglyceraldehyde enters the pathway, hence, dihydroxyacetonephosphate also gets converted to 3-phosphoglyceraldehyde. This reaction is catalyzed by the enzyme phosphotriose isomerase.
   Glycerol can also enter glycolytic pathway at this point.
6. In the next step, in presence of NAD^+ and inorganic phosphate (Pi), glyceraldehyde-3-phosphate dehydrogenase oxidizes 3-phosphoglyceraldehyde to 1,3-bisphosphoglycerate.
7. In the next step, 1,3-bisphosphoglycerate is converted to 3-phosphoglycerate. This reaction is catalyzed by the enzyme phosphoglycerate kinase. In this reaction, there is a generation of ATP. It is called as substrate level production of ATP (i.e. substrate level phosphorylation).
   In RBCs this conversion occurs via formation of 2,3-BPG, where no formation of ATP occurs in this step.
   Arsenate inhibits phosphorylation at this step and thus, ATP production.
8. Thereafter, 3-phosphoglycerate is converted to 2-phosphoglycerate by the enzyme phosphoglycerate mutase.
9. In the next step, enolase converts 2-phosphoglycerate to a high-energy compound, called phosphoenolpyruvate.
   Enolase requires Mg^{2+} for its activity but is inhibited by fluoride. Since fluoride inhibits this reaction, therefore, fluoride is added to the blood, as a preservative, in glucose estimation.
10. Subsequently, phosphoenolpyruvate is converted to pyruvate, irreversibly. This reaction is catalyzed by the enzyme pyruvate kinase, which, in turn, phosphorylates ADP to ATP.
    Deficiency of pyruvate kinase leads to hemolytic anemia.
11. Pyruvic acid is reduced to lactic acid under anaerobic conditions or is oxidized to acetyl CoA under aerobic conditions.
    - **Under anaerobic conditions** pyruvate is **reduced to lactate**. This reaction is catalyzed by the enzyme **lactate dehydrogenase**, which requires NADH.



Tissues such as skeletal muscle, when function under hypoxic conditions, produce large amount of lactic acid.

- **Under aerobic conditions**, pyruvate is transported into mitochondria, where it is **oxidatively decarboxylated to acetyl CoA** by the enzyme **pyruvate dehydrogenase complex** (Fig. 29.3).

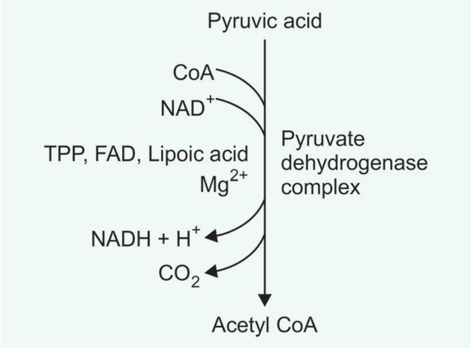


Fig. 29.3: Conversion of pyruvic acid to acetyl CoA

Pyruvate dehydrogenase complex requires five coenzymes, i.e. **TPP, CoA, NAD, FAD** and **lipoic acid**. Insulin stimulates its activity, whereas acetyl CoA, NADH and increased ATP/ADP ratio inhibit it.

Bioenergetics (ATP production) of Glycolysis

Number of ATP produced during glycolysis vary, depending upon the condition.

- **Under anaerobic condition**, conversion of 1,3-bisphosphoglycerate to 3-phosphoglycerate and phosphoenolpyruvate to pyruvate, each produces one ATP, a total of 2 ATP is produced per molecule of a triose, or 4 molecules of ATP per molecule of hexose (Fig. 29.4).

  As 2 ATP are used in the initial two reactions of the process, i.e. in the conversion of glucose to glucose-6-phosphate and fructose-6-phosphate to fructose-

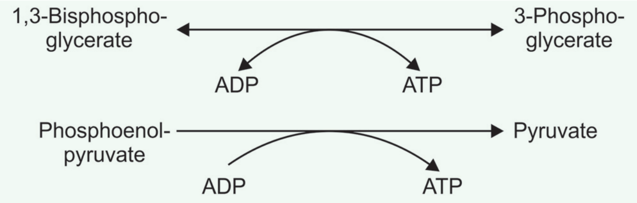

1,3-Bisphospho-glycerate
3-Phospho-glycerate
ADP
ATP
Phosphoenol-pyruvate
Pyruvate
ADP
ATP

Fig. 29.4: ATP producing steps in anaerobic glycolysis

1,6-bisphosphate, the net energy yield per molecule of glucose is only 2 ATP.

- **Under aerobic conditions** NADH, which is produced during the conversion of 3-phosphoglycerte to 1,3-bisphosphoglycerate, can enter the electron transport chain and release 2.5 ATP, thus, additionally, 5 more ATP are produced per molecule of glucose. Therefore, a total yield under aerobic conditions is 9 ATP while the net yield is 7 ATP.

Biomedical Importance of Glycolysis

In the liver and other tissues, anaerobic glycolysis is the main source of ATP. In the muscle, anaerobic glycolysis provides energy during exercise.

Regulation of Glycolysis

There are three irreversible steps which are catalyzed by hexokinase, phosphofructokinase and pyruvate kinase. These are also the sites of the regulation of glycolysis.

Insulin stimulates these enzymes and increases utilization of glucose by glycolysis. On the other hand, **glucagon inhibits** this process.

TRICARBOXYLIC ACID CYCLE

Tricarboxylic acid cycle (TCA cycle), also called **citric acid cycle** or **Krebs cycle**, is a process of the **oxidation of acetyl CoA** (active acetate, produced under aerobic glycolysis) to **CO_2** and **H_2O**.

During the course of oxidation of acetyl CoA reducing equivalents are produced, which enter respiratory chain and generate large amount of ATP.

Outline of TCA Cycle

Reactions of the TCA cycle are outlined in Fig. 29.5.

1. In the first step, **acetyl CoA** (formed from pyruvate under aerobic condition) combines with **oxaloacetate** and forms citric acid (a tricarboxylic acid).

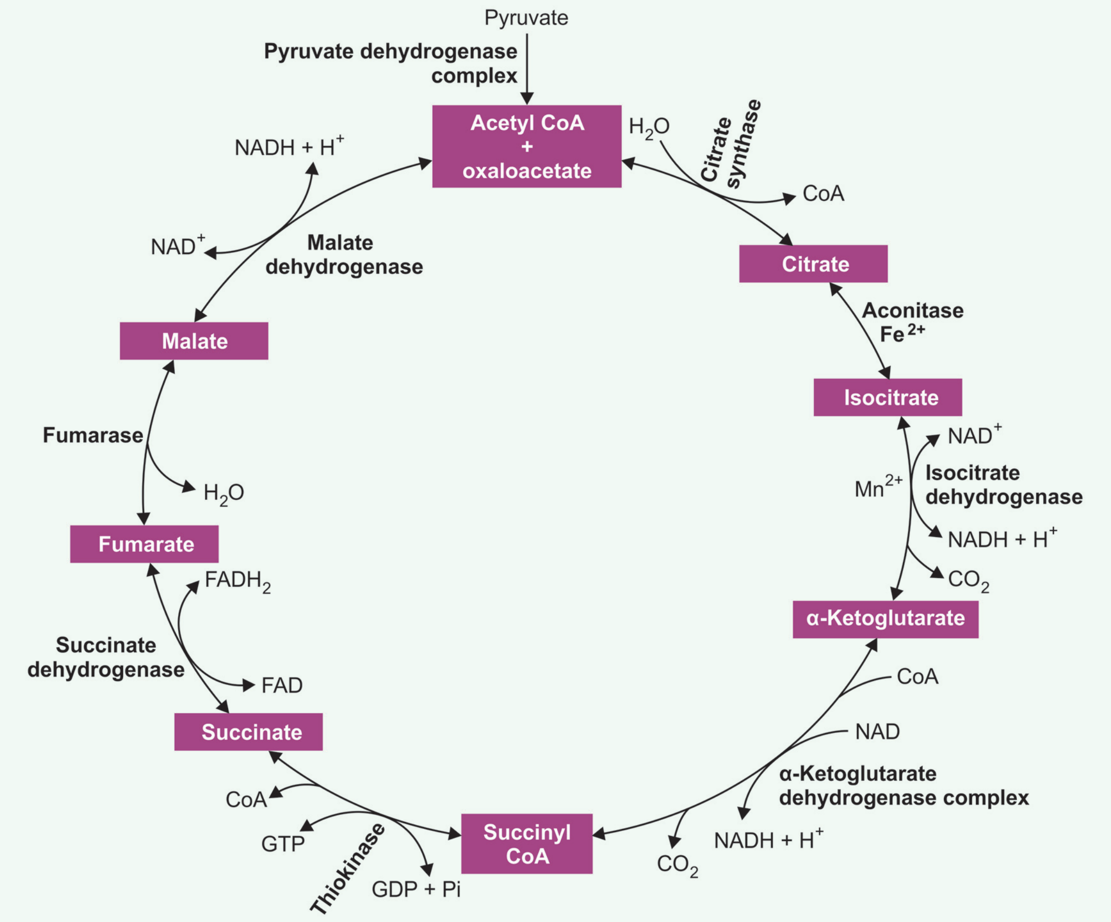

Pyruvate
Pyruvate dehydrogenase complex
Acetyl CoA + oxaloacetate
H2O
Citrate synthase
CoA
Citrate
Aconitase Fe2+
Isocitrate
NAD+
Mn2+
Isocitrate dehydrogenase
NADH + H+
CO2
α-Ketoglutarate
CoA
NAD
α-Ketoglutarate dehydrogenase complex
NADH + H+
CO2
Succinyl CoA
GDP + Pi
Thiokinase
GTP
CoA
Succinate
FAD
Succinate dehydrogenase
FADH2
Fumarate
H2O
Fumarase
Malate
NAD+
Malate dehydrogenase
NADH + H+

Fig. 29.5: Outline of citric acid cycle

This reaction is catalyzed by the enzyme **citrate synthase**, also called the **condensing enzyme**.

2. **Citrate** is then rearranged to **cis-aconitate**, which is subsequently changed to isocitrate. Both of these steps are catalyzed by **aconitase**.
   Conversion of citrate to isocitrate is **inhibited by fluoroacetate**.
3. In the next step, in the presence of **isocitrate dehydrogenase**, **isocitrate** is converted to oxalosuccinate which is subsequently decarboxylated to α-ketoglutarate.
4. Thereafter, α-ketoglutarate undergoes oxidative decarboxylation and gets converted to **succinyl CoA**. This reaction is similar to the conversion of pyruvate to acetyl CoA. **α-Ketoglutarate dehydrogenase complex**, which also requires five coenzymes, i.e. TPP, NAD^+, FAD, coenzyme A and lipoic acid, catalyzes this reaction.
5. In the next step, **succinyl CoA** is converted to **succinate**, by the enzyme **succinate thiokinase**.
   During this reaction, a molecule of GTP is formed. This is known as **substrate level phosphorylation, as a high-energy** molecule is formed at the substrate level.
6. Thereafter, **succinate** is converted to **fumarate**, by the enzyme **succinate dehydrogenase**.
   Due to structural similarities between malonate and succinate, **malonate inhibits** succinate dehydrogenase, **competitively**.
7. In the next step, with the addition of a molecule of water, by the enzyme **fumarase** (fumarate hydratase), **fumarate** is converted to **L-malate**.
8. Finally, **malate dehydrogenase**, in the presence of NAD^+, converts **malate** to **oxaloacetate**.

Bioenergetics (ATP Production) of TCA Cycle

As a result of oxidation of one molecule of acetyl CoA in the Krebs cycle, three molecules of NAD^+ and one molecule of FAD are reduced.

Reducing equivalents (from NADH + H^+) enter respiratory chain at three different levels and result in the production of 2.5 ATP at each level. Similarly, $FADH_2$ yields 1.5 ATP. Besides, there is also substrate level production of GTP.

Thus, **total ATP yield**, per molecule of acetyl CoA, is **10 ATP** (Table 29.1).

As mentioned above, conversion of pyruvate to acetyl CoA also generates NADH + H^+ and gives 2.5 ATP. Thus, total number of ATP produced from oxidation of pyruvate is 12.5.

Table 29.1 Energy producing steps in citric acid cycle

Reaction	Reducing equivalents produced as	Number of ATP produced
Isocitrate → α-Ketoglutarate	NADH + H^+	2.5
α-Ketoglutarate → Succinyl CoA	NADH + H^+	2.5
Succinyl CoA → Succinate	—	1
Succinate → Fumarate	$FADH_2$	1.5
Malate → Oxaloacetate	NADH + H^+	2.5
	Total	**10**

Since 2 molecules of pyruvate are formed from one molecule of glucose, therefore, in addition to energy yield during aerobic glycolysis (7 ATP) a molecule of glucose also produces 25 ATP via the Krebs cycle. Thus, a total of 32 ATP are obtained when a molecule of glucose is completely oxidized to CO_2 and H_2O under aerobic conditions, i.e. via glycolysis and Krebs cycle.

Biomedical Importance of TCA Cycle

Citric acid cycle has a **dual role**, i.e. it is important in oxidation as well as synthetic processes. It is, thus, **amphibolic** in nature.

- It is **catabolic** for the oxidation of carbohydrates, lipids and proteins, as they are completely oxidized to CO_2 and H_2O, and release energy.
- It is also important in the **anabolic** reactions, as various intermediates of the cycle can be used for the biosynthesis of the nonessential amino acids.
- Various intermediates of the cycle are also potentially glucogenic and thus, can give rise to glucose in liver and kidney.

Regulation of TCA Cycle

TCA cycle is regulated by the availability of acetyl CoA, oxaloacetate and NADH. Citrate synthase, isocitrate dehydrogenase and α-ketoglutarate dehydrogenase are the rate limiting enzymes of the TCA cycle.

GLUCONEOGENESIS

Gluconeogenesis (neoglucogenesis) is the process of the **formation of glucose** from various **non-carbohydrate sources** such as the glucogenic amino acids, lactate, glycerol, or propionate.

- Gluconeogenesis takes place in the **fasting state** or on a low carbohydrate diet, particularly, in the liver and some other tissues, which are solely dependent on glucose for their energy demand. It does not take place in muscle.

- Gluconeogenesis thus, enables **maintenance of blood glucose** when all the dietary glucose has been absorbed and oxidized. This process is essential since blood glucose level has to be maintained, to support metabolism of the tissues that use glucose as the primary substrate such as the brain, red blood cells and lens.

Gluconeogenesis is said to be the reversal of glycolysis but truly it is not a reversal of glycolysis, since there are three irreversible reactions in the glycolytic pathway (Fig. 29.6).

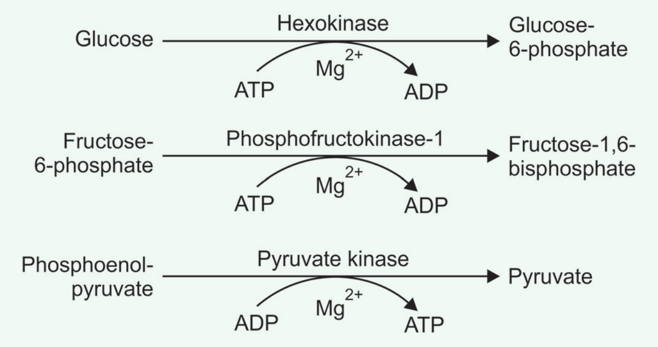

Glucose
Hexokinase
Glucose-6-phosphate
Mg^{2+}
ATP
ADP
Fructose-6-phosphate
Phosphofructokinase-1
Fructose-1,6-bisphosphate
Mg^{2+}
ATP
ADP
Phosphoenol-pyruvate
Pyruvate kinase
Pyruvate
Mg^{2+}
ADP
ATP

Fig. 29.6: Irreversible steps in glycolysis

Outline of Gluconeogenesis

A. Reversal of Glycolysis

It includes the following set of reactions:

1. **Conversion of pyruvate to phosphoenolpyruvate**
   - In the reversal of the pyruvate to phosphoenolpyruvate, pyruvate is first converted to oxaloacetate by the enzyme **pyruvate carboxylase**, in mitochondria (Fig. 29.7).

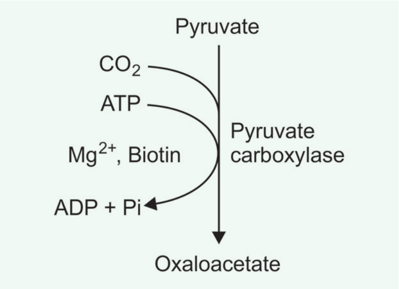

Pyruvate
CO_2
ATP
Mg^{2+}, Biotin
ADP + Pi
Pyruvate carboxylase
Oxaloacetate

Fig. 29.7: Conversion of pyruvate to oxaloacetate

   - Thereafter, malate dehydrogenase converts oxaloacetate to malate, which is freely transported across the mitochondrial membrane. Malate thus, comes out from the mitochondria to the cytosol where it is reconverted back to oxaloacetate by the same enzyme. This also reduces NAD^+ to $NADH+H^+$. This enzyme thus, not only transports oxaloacetate (via malate) but also reducing equivalents from mitochondria into cytosol. Reactions of gluconeogenesis, thus, occur in the cytosol as well as mitochondria.
   - In the next step, **oxaloacetate** is converted to **phosphoenolpyruvate**, by the enzyme **phosphoenolpyruvate carboxykinase** (PEP-carboxykinase; Fig. 29.8).

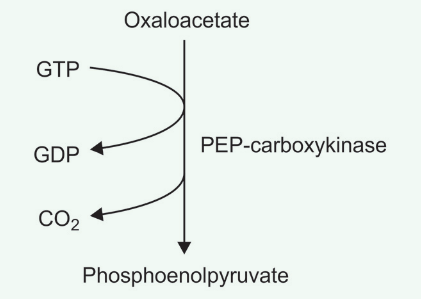

Oxaloacetate
GTP
GDP
CO_2
PEP-carboxykinase
Phosphoenolpyruvate

Fig. 29.8: Conversion of oxaloacetate to phosphoenolpyruvate

2. **Conversion of fructose-1,6-bisphosphate to fructose-6-phosphate**
   - Fructose-1,6-bisphosphate is converted to fructose-6-phosphate by the enzyme **fructose-1,6-bisphosphatase** (Fig. 29.9).

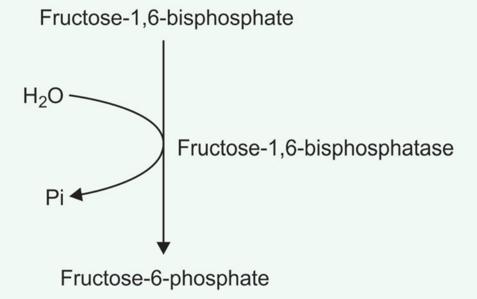

Fructose-1,6-bisphosphate
H_2O
Pi
Fructose-1,6-bisphosphatase
Fructose-6-phosphate

Fig. 29.9: Conversion of fructose-1,6-bis-P to fructose-6-P

3. **Conversion of glucose-6-phosphate to glucose**
   - Glucose-6-phosphate is converted to glucose by the enzyme **glucose-6-phosphatase**. This enzyme is not found in the muscle and adipose tissue (Fig. 29.10).

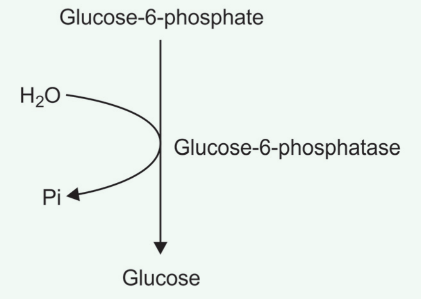

Glucose-6-phosphate
H_2O
Pi
Glucose-6-phosphatase
Glucose

Fig. 29.10: Conversion of glucose-6-P to glucose

B. Conversion of Lactate to Glucose

Lactate is produced during the process of anaerobic glycolysis. For its conversion to glucose, firstly, **lactate** is oxidized to **pyruvate**. This reaction is catalyzed by the enzyme **lactate dehydrogenase**, which requires NAD^+ (Fig. 29.11).

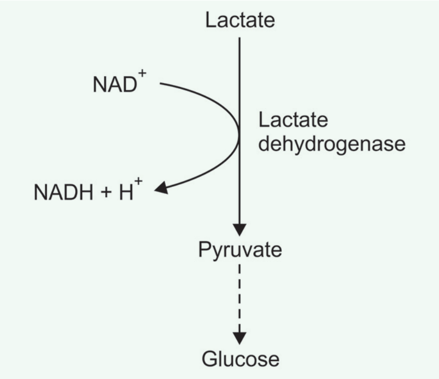

Lactate
NAD+
Lactate dehydrogenase
NADH + H+
Pyruvate
Glucose

Fig. 29.11: Conversion of lactate to glucose

Subsequently, pyruvate is converted to glucose by the **reversal of the glycolytic reactions**.

C. Conversion of Glucogenic Amino Acids to Glucose

Various glucogenic amino acids transfer their α-amino group by transamination and release carbon skeletons which form intermediates of the citric acid cycle (Table 29.2).

Table 29.2 Conversion of glucogenic amino acids to the citric acid cycle intermediates

Amino acid	Citric acid cycle intermediate formed
Gly, Ala, Ser, Thr, Cys, Trp and HO-Pro	Pyruvate
Arg, His, Glu, Gln and Pro	α-Ketoglutarate
Phe and Tyr	Fumarate
Val, Ile and Met	Succinyl CoA

These citric acid cycle intermediates, in turn, can form glucose by the reversal of the glycolytic reactions, via pyruvate.

D. Conversion of Glycerol to Glucose

Glycerol is produced as a result of lipolysis in the adipose tissue. **Glycerol kinase** converts it to **α-glycerolphosphate** in the liver, which is later reduced by a dehydrogenase to **dihydroxyacetone phosphate** and can enter glycolysis at this point (Fig. 29.12).

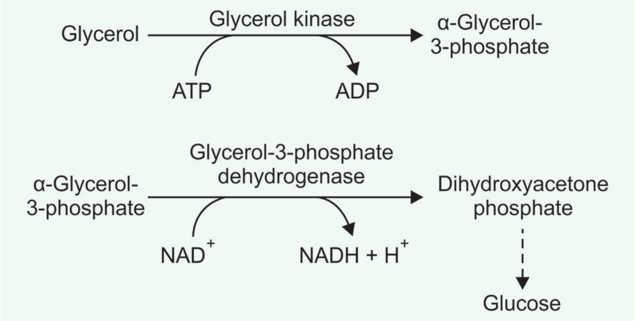

Glycerol
Glycerol kinase
α-Glycerol-3-phosphate
ATP
ADP
Glycerol-3-phosphate dehydrogenase
α-Glycerol-3-phosphate
Dihydroxyacetone phosphate
NAD+
NADH + H+
Glucose

Fig. 29.12: Conversion of glycerol to glucose

E. Conversion of Propionyl CoA to Glucose

Oxidation of fatty acids containing odd number of carbon atoms, in addition to acetyl CoA, also produces a molecule of **propionyl CoA**, particularly, in ruminants. In human beings, propionyl CoA is obtained from Ile, Val and Thr. It is converted to **D-methylmalonyl CoA** by **propionyl CoA carboxylase**. D-Methylmalonyl CoA is then converted to **L-methylmalonyl CoA** and finally by an isomerase to **succinyl CoA** which is an intermediate of the citric acid cycle. Succinyl CoA, in turn, can be converted to glucose via pyruvate (Fig. 29.13).

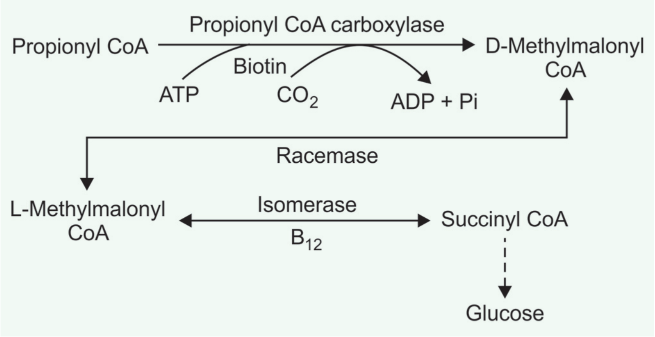

Propionyl CoA
Propionyl CoA carboxylase
D-Methylmalonyl CoA
Biotin
ATP
CO2
ADP + Pi
Racemase
L-Methylmalonyl CoA
Isomerase
B12
Succinyl CoA
Glucose

Fig. 29.13: Conversion of propionyl CoA to glucose

Biomedical Importance and Bioenergetics of Gluconeogenesis

Gluconeogenesis occurs in the fasting state or on a low carbohydrate diet in the liver and other tissues, which are solely dependent on glucose for their energy demand. Thus, it enables the maintenance of blood glucose, when it is in short supply.

Regulation of Gluconeogenesis

Gluconeogenesis is regulated by the enzymes pyruvate carboxylase, phosphoenolpyruvate carboxykinase, fructose-1,6-bisphosphatase and glucose-6-phosphatase, which catalyse the reversal of the irreversible steps of glycolysis.

GLYCOGEN METABOLISM

GLYCOGENESIS

Glycogenesis (**glycogen synthesis**) is the process of the **conversion of glucose to glycogen**. Although, it is operative in several tissues, liver and muscle are the main organs for the synthesis of glycogen. Glycogenesis is stimulated by insulin.

Various reactions of glycogenesis are outlined in Fig. 29.14.

1. **Glucose** is first activated (phosphorylated) to **glucose-6-phosphate**.

   In the liver, **glucokinase** converts most of the glucose into glucose-6-phosphate, in the fed state. This is an inducible enzyme and has greater specificity for its substrate. In the muscle and other tissues, this reaction is catalyzed by **hexokinase**.
2. **Glucose-6-phosphate** is isomerised to **glucose-1-phosphate**, by the enzyme **phosphoglucomutase**.
3. **Glucose-1-phosphate** reacts with UTP and gets converted to **uridinediphosphate glucose** (UDP-Glu or UDPG). The reaction is catalyzed by **UDPG pyrophosphorylase** (glucose-1-phosphate uridyl-transferase).

   Pyrophosphate, so released during this process, is immediately hydrolyzed to two molecules of **inorganic phosphate**, by **pyrophosphatase**.
4. From **UDP-Glu**, glucose is transferred to the **glycogen primer** (i.e. the preformed oligosaccharide). This reaction is catalyzed by the enzyme **glycogen synthase**.

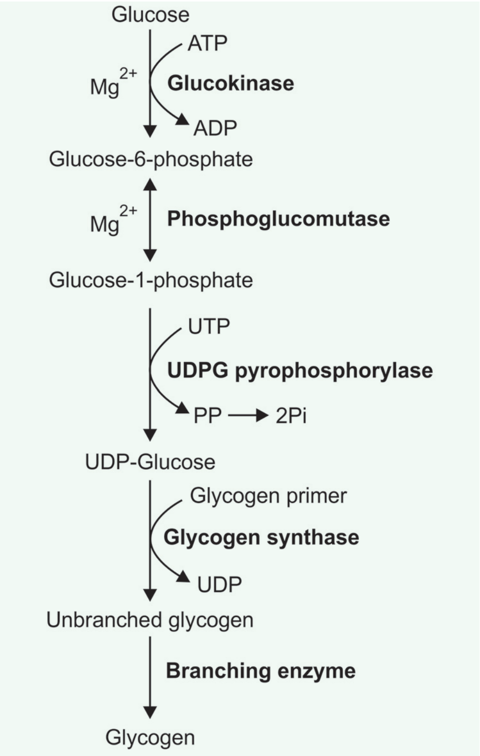

Glucose
ATP
Mg^{2+}
Glucokinase
ADP
Glucose-6-phosphate
Mg^{2+}
Phosphoglucomutase
Glucose-1-phosphate
UTP
UDPG pyrophosphorylase
PP → 2Pi
UDP-Glucose
Glycogen primer
Glycogen synthase
UDP
Unbranched glycogen
Branching enzyme
Glycogen

Fig. 29.14: Outline of glycogenesis

The incoming glucose is linked to the primer at the non-reducing end by 1,4-α-glycosidic linkage and results in elongation of the pre-existing branch. This results in the formation of an **unbranched glycogen**. Both, glycogen primer and UDP-Glu are the substrates for glycogen synthase, which is a key enzyme of glycogenesis.

5. Once the straight chain containing nearly 11 glucose residues is formed, **branching enzyme** removes a block of 7–8 glycosyl residues from the growing chain and transfers it to the neighboring chain (Fig. 29.15).

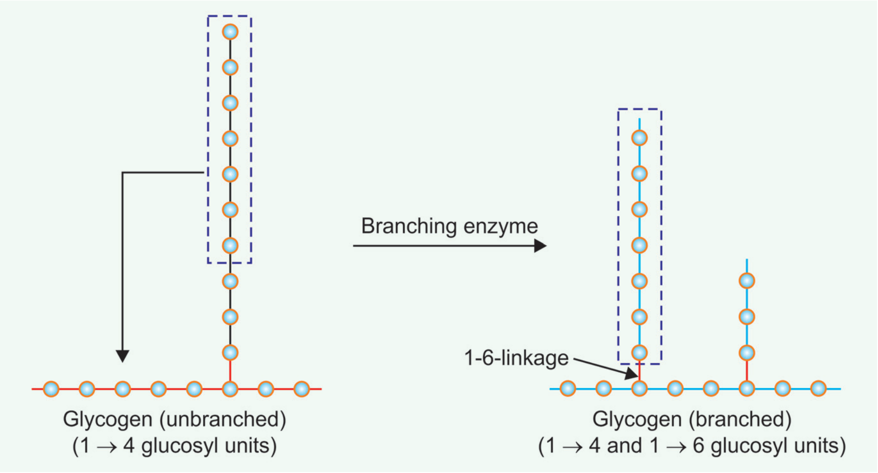

Branching enzyme
1-6-linkage
Glycogen (unbranched)
(1 → 4 glucosyl units)
Glycogen (branched)
(1 → 4 and 1 → 6 glucosyl units)

Fig. 29.15: Formation of a branch point by the branching enzyme during glycogenesis

This branch point again grows with the addition of the glucose molecules at the 1→4 linkage. This, in turn, results in the formation of a highly branched glycogen structure.

GLYCOGENOLYSIS

Glycogenolysis (**glycogen breakdown**) is the process of the conversion of **glycogen** to either **glucose-6-phosphate** in muscle or **free glucose** in **liver** and kidney (Fig. 29.16).

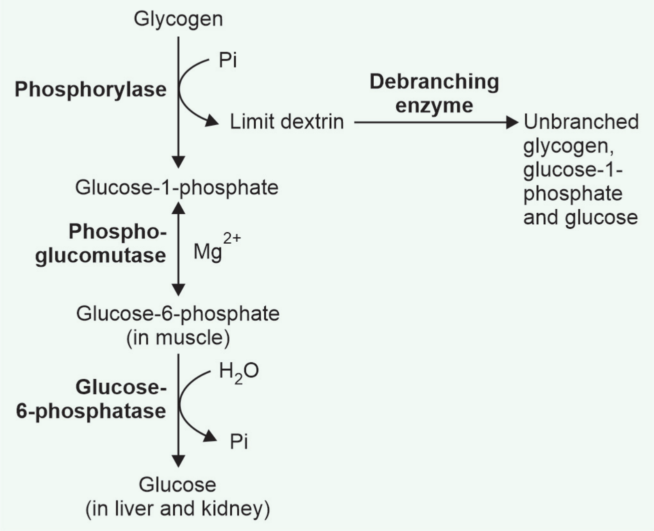

Glycogen
Pi
Phosphorylase
Limit dextrin
Debranching enzyme
Unbranched glycogen, glucose-1-phosphate and glucose
Glucose-1-phosphate
Phospho-glucomutase
Mg2+
Glucose-6-phosphate (in muscle)
H2O
Glucose-6-phosphatase
Pi
Glucose (in liver and kidney)

Fig. 29.16: Outline of glycogenolysis

1. In the first step, glucose molecules are sequentially removed from **glycogen** as **glucose-1-phosphate**. This reaction is catalyzed by the enzyme phosphorylase. It is the rate limiting step of this pathway.

   Phosphorylase hydrolyzes the α-1,4-glycosidic bonds and removes glucose units as glucose-1-phosphate, until nearly four glucose residues are left. After the action of phosphorylase, **glycogen** is partially hydrolyzed, leaving **limit dextrin**.
2. Limit dextrin is further hydrolyzed by the debranching enzyme to unbranched glycogen and finally to glucose-1-phosphate and glucose.

   Debranching enzyme is a bifunctional enzyme. Its first unit has **glycosyltransferase activity**, which removes a strand of three glucosyl residues from the four glucosyl-residues of the branch and attaches the same to the nonreducing end of another branch. This reaction forms a new α(1→4) linkage and thus, three more glucose units become available for the phosphorylase reaction (Fig. 29.17).

   The α(1→6) bond, which is linking the remaining glucosyl residue on the branch point to the main chain, is hydrolyzed by the second unit of the debranching enzyme that is referred to as **amylo-α-1,6-glucosidase**. This glucosyl residue is released as free glucose, rather than as glucose-1-phosphate, leaving the unbranched glycogen molecule.

   Cooperative and repetitive actions of phosphorylase and debranching enzyme result in complete hydrolysis of glycogen.
3. **Glucose-1-phosphate**, produced as a result of the action of phosphorylase, is converted to **glucose-6-phosphate** by the enzyme **phosphoglucomutase**.

   Glucose-6-phosphate is the end product of glycogen breakdown **in muscle**, where it is used in the glycolytic pathway to liberate energy that is required during exercise. Thus, glycogenolysis is followed by glycolysis, due to the absence of the enzyme glucose-6-phosphatase in the muscle.

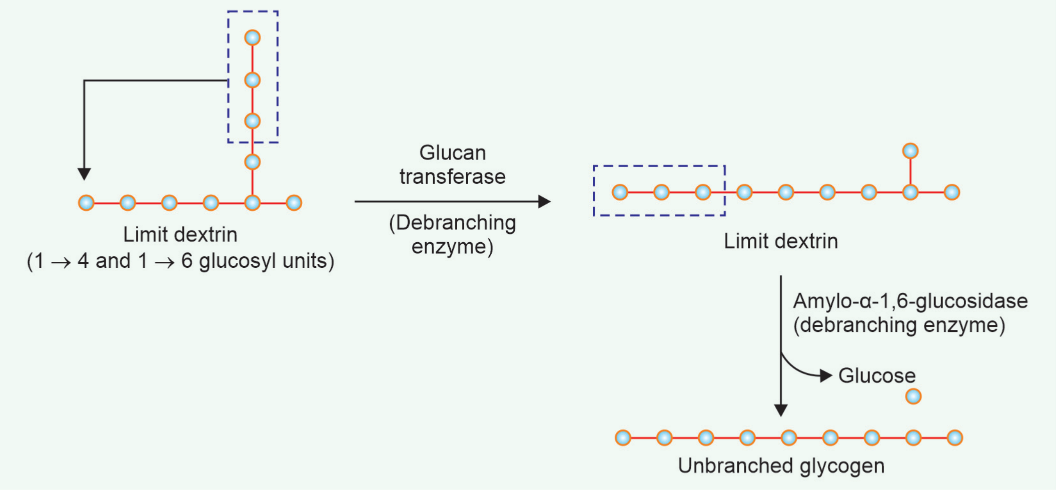

Limit dextrin
(1 → 4 and 1 → 6 glucosyl units)
Glucan transferase
(Debranching enzyme)
Limit dextrin
Amylo-α-1,6-glucosidase
(debranching enzyme)
Glucose
Unbranched glycogen

Fig. 29.17: Removal of a branch point by the debranching enzyme during glycogenolysis

4. **In the liver and kidney**, glucose-6-phosphate is further hydrolyzed to **glucose**. This reaction is catalyzed by **glucose-6-phosphatase**.

Biomedical Importance of Glycogen Metabolism

Glycogen, which is stored **in the liver**, is converted to free glucose and is used to **maintain blood glucose level** in a hypoglycemic state. On the other hand, **in the muscle**, glycogen is used for providing energy during exercise.

Regulation of Glycogen Metabolism

Glycogen metabolism in the liver is controlled by glucagon while in the muscle and other tissues, it is controlled by insulin and norepinephrine/epinephrine.

Glycogenesis is regulated by glycogen synthase, whereas glycogenolysis is regulated by glycogen phosphorylase. Both the enzymes are under allosteric control and are regulated by the availability of ATP and glucose-6-phosphate, reversibly.

Glycogen Storage Diseases

This is a group of inherited disorders that are associated with glycogen metabolism, where an abnormal type or quantity of the glycogen is deposited in different tissues. According to the deficiency of the enzyme involved, there are several types of glycogen storage diseases. Though more than 15 types of glycogen storage disorders are known to occur, three of these are most common in children. These are referred to as von Gierke disease (type I), Cori disease or Forbes disease (type III) and Andersen disease (type IV; Table 29.3).

HEXOSE MONOPHOSPHATE SHUNT

Hexose monophosphate shunt (HMP shunt), also called **pentose phosphate pathway**, is the second major pathway for the metabolism of glucose. Enzymes for this pathway are localized in the cytosol, while reducing equivalents are accepted by $NADP^+$ instead of NAD^+. This pathway is operative in many tissues such as liver, erythrocytes, lactating mammary glands, testes and adipose tissue. Rate of the HMP shunt reactions is increased by insulin.

In the overall process, six molecules of glucose are utilized to give six molecules of CO_2 and six molecules of pentoses. The pentoses are subsequently, rearranged to give four molecules of fructose-6-phosphate and two molecules of glyceraldehyde-3-phosphate. Two trioses also form a hexose by the reversal of glycolysis thus, regenerating five molecules of hexoses (Fig. 29.18).

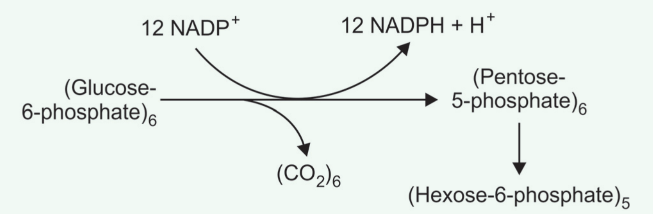


Fig. 29.18: HMP shunt: Overall process

Table 29.3 Some of the glycogen storage diseases

Type	Glycogen storage disease	Deficient enzyme	Features
I	von Gierke disease	Glucose-6-phosphatase	Liver, renal and intestinal epithelial cells are loaded with glycogen. There also occurs hypoglycemia, lactic acidosis, ketosis, hyperlipidemia and hyperuricemia
II	Pompe's disease	Lysosomal acid maltase	Accumulation of glycogen in the lysosomes. It is fatal and may result in heart failure
III	Cori disease or Forbes disease	Debranching enzyme	Accumulation of the limit dextrin in liver, muscle and heart.
IV	Andersen disease	Branching enzyme	Accumulation of amylopectin, hence also referred to as amylopectinosis. Death may occur due to liver or cardiac failure within first year of life.
V	McArdle's syndrome	Muscle phosphorylase	Muscle has abnormally high glycogen content. There is diminished exercise tolerance with little or no lactate in blood after exercise
VI	Her's disease	Liver phosphorylase	High glycogen content in liver. There is also a tendency towards hypoglycemia
VII	Tarui disease	Phosphofructokinase	As in type V, but there is also a possibility of hemolytic anemia
VIII	—	Liver phosphorylase kinase	As in type VI

Outline of HMP Shunt

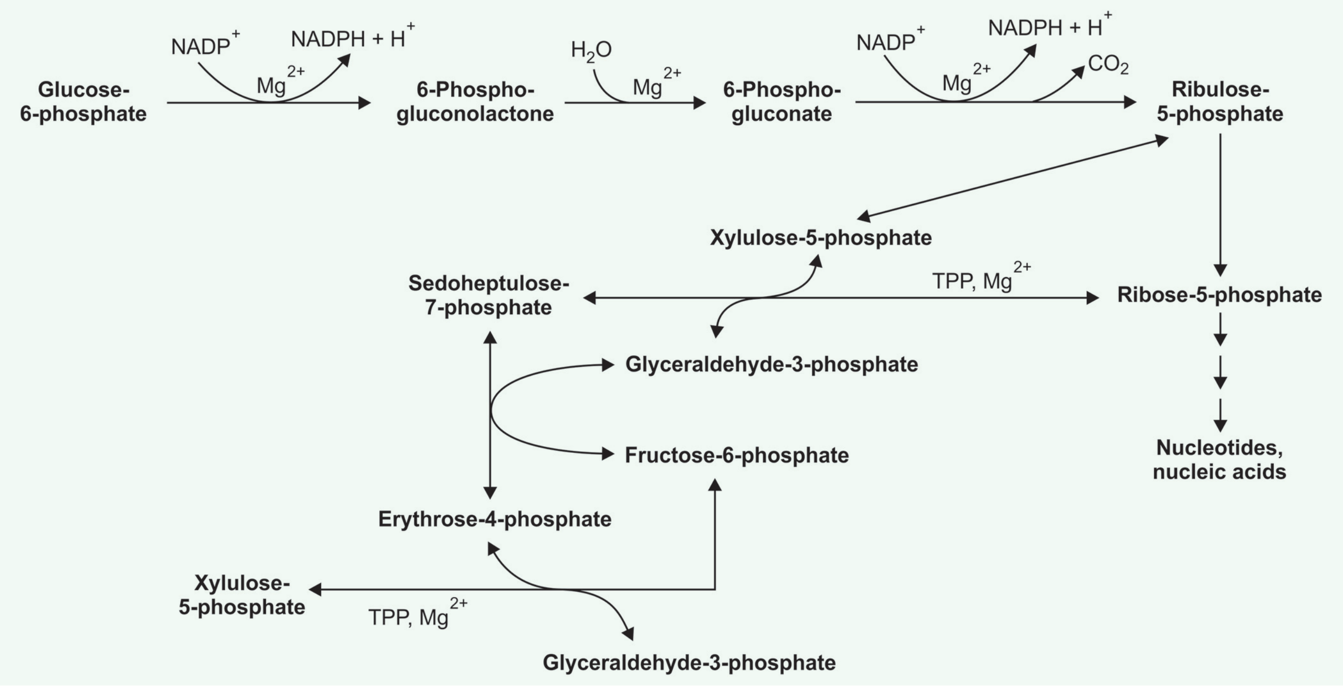
Reactions of the HMP shunt are outlined in Fig. 29.19.

1. Firstly, **glucose** is activated to **glucose-6-phosphate** by the enzyme hexokinase.
2. Thereafter, **glucose-6-phosphate** is changed to **6-phosphogluconolactone** and, subsequently, to **6-phosphogluconate** by the enzyme **glucose-6-phosphate dehydrogenase**. This enzyme is induced by insulin. During this process $NADP^+$ is reduced to $NADPH+H^+$.
3. In the next reaction, a hexose is converted to a pentose. In this process, **6-phosphogluconate** is oxidized by the enzyme **6-phosphogluconate dehydrogenase**, to form an intermediate called **3-keto-6-phosphogluconate**. This reaction also generates $NADPH + H^+$.
4. Subsequently, by the enzyme **6-phosphogluconate decarboxylase**, a carbon is removed from **3-keto-6-phosphogluconate** as CO_2, leaving a **pentose** called **ribulose-5-phosphate**.

   These two enzymes, i.e. glucose-6-phosphate dehydrogenase and 6-phosphogluconate dehydrogenase, are the regulatory enzymes of this shunt.
5. In the second stage of the shunt, ribulose-5-phosphate changes to other pentoses.
   - **Ribulose-5-phosphate** is changed to **ribose-5-phosphate** by the enzyme **ribose-5-phosphate ketoisomerase**.
   - It is also converted to **xylulose-5-phosphate** by the enzyme **ribulose-5-phosphate epimerase**.
6. In the next reaction, carbon skeletons of the two pentoses are rearranged.
   - Firstly, **transketolase** transfers a ketol group (the first 2 carbon atoms) from **xylulose-5-phosphate** to **ribose-5-phosphate**, forming **sedoheptulose-7-phosphate** and **glyceraldehyde-3-phosphate**. Transketolase requires TPP as a coenzyme.
   - Subsequently, an aldol group (3 carbons moiety) is transferred by the enzyme **transaldolase**, from **sedoheptulose-7-phosphate** to **glyceraldehyde-3-phosphate**, forming **fructose-6-phosphate** and **erythrose-4-phosphate**.
7. Thereafter, **transketolase** further transfers a ketol group (2 carbons moiety) from **xylulose-5-phosphate** to **erythrose-4-phosphate**, forming **fructose-6-phosphate** and **glyceraldehyde-3-phosphate**.

Biomedical Importance of HMP Shunt

- **In the liver:** In the liver HMP shunt serves as a source of NADPH and pentoses.



Glucose-6-phosphate
NADP+
NADPH + H+
Mg2+
6-Phospho-gluconolactone
H2O
Mg2+
6-Phospho-gluconate
NADP+
NADPH + H+
Mg2+
CO2
Ribulose-5-phosphate
Xylulose-5-phosphate
Sedoheptulose-7-phosphate
TPP, Mg2+
Ribose-5-phosphate
Glyceraldehyde-3-phosphate
Fructose-6-phosphate
Nucleotides, nucleic acids
Erythrose-4-phosphate
Xylulose-5-phosphate
TPP, Mg2+
Glyceraldehyde-3-phosphate

Fig. 29.19: Outline of HMP shunt

- **Generation of NADPH:** Two molecules of $NADP^+$ are reduced to NADPH per molecule of glucose-6-phosphate, in the pentose phosphate pathway. **NADPH**, so produced, is used for the various **anabolic reactions** such as synthesis of fatty acids and cholesterol.
- **Source of pentoses:** Pentose phosphate pathway is also important source of pentoses which are required for the **synthesis of nucleic acids** (Fig. 29.20).

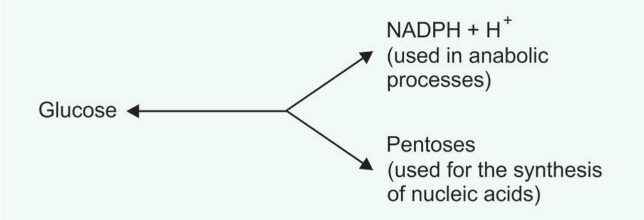

NADPH + H+
(used in anabolic processes)
Glucose
Pentoses
(used for the synthesis of nucleic acids)

Fig. 29.20: Biomedical importance of HMP shunt in the liver

- **In the erythrocytes:** NADPH is utilized by glutathione reductase in the erythrocytes to maintain the reduced state of glutathione and prevent their hemolysis. This reaction is important for the detoxification of H_2O_2 which is produced in the erythrocytes. If accumulates, H_2O_2 decreases their life span and causes hemolysis (Fig. 29.21).

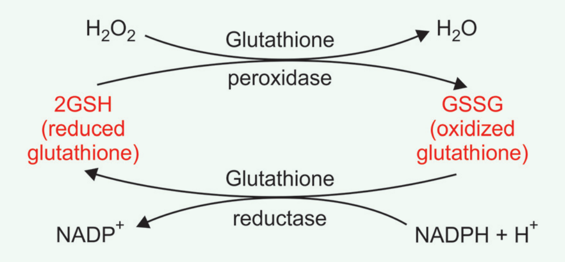


Fig. 29.21: Biomedical importance of HMP shunt in erythrocytes

HORMONAL REGULATION OF BLOOD GLUCOSE

Blood glucose level is maintained within the **normal** physiological **range** of **60–90 mg per 100 mL** in the fasting (post-absorptive) state by various **hormones**, e.g. insulin lowers blood glucose while various other hormones increase blood glucose level (Fig. 29.22).

By Insulin

Insulin is the only hormone which has hypoglycemic action. It is released into the blood stream under the direct influence of hyperglycemia. It is a polypeptide containing 2 chains and is produced by **β-cells of the islets of Langerhans** of the pancreas.

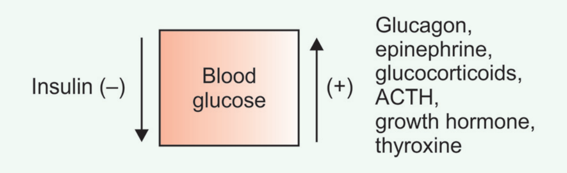


Fig. 29.22: Regulation of blood glucose by hormones

- Insulin **lowers blood glucose** concentration by increasing uptake of glucose by the extrahepatic tissues. Insulin also **promotes glycolysis** and **glycogenesis**, both in the liver and the muscle.
- At the same time, insulin also **suppresses glycogenolysis** in the liver and the kidney.
- Besides, insulin also suppresses **gluconeogenesis**.
- Insulin **stimulates the synthesis of various enzymes** such as glucose-6-phosphate dehydrogenase, 6-phosphogluconate dehydrogenase, ATP-citrate lyase, etc.
- It also **represses the synthesis** of pyruvate carboxylase, PEP-carboxykinase and fructose-1,6-bisphosphatase.
- Insulin also **promotes transport of amino acids** across the cell membrane and, thus, **stimulates protein synthesis**.
- It also **inhibits ketogenesis** and **promotes lipogenesis**.

By Hormones which have Hyperglycemic Action

Several hormones have hyperglycemic action. These are called diabetogenic hormones and include **glucagon**, **epinephrine**, **glucocorticoids**, **growth hormone** and **thyroxine**.

Glucagon

Glucagon is produced by **α-cells** of the **islets of Langerhans** of pancreas. Its synthesis is stimulated under the influence of hypoglycemia. It acts only in the liver and **promotes glycogenolysis.** It also promotes **gluconeogenesis** from amino acids and lactate.

Epinephrine

Epinephrine, also called **adrenaline**, is secreted by **adrenal medulla**. It is a hormone in the first line of defense against hypoglycemia. It is a derivative of tyrosine.

Epinephrine **promotes glycogenolysis**, both in the liver and muscle. Epinephrine also promotes **gluconeo-**

genesis in the liver. It **also inhibits release of insulin** from the pancreas, and decreases transport and utilization of glucose in different tissues. At the same time, epinephrine also **stimulates secretion of glucagon**.

Glucocorticoids

Glucocorticoids are steroids secreted by **adrenal cortex**. They are antagonist to insulin. Cortisol is a major glucocorticoid present in blood. It **stimulates protein catabolism** in the **muscle** and **promotes gluconeogenesis** in the **liver**. It also **stimulates lipolysis** and **inhibits glucose oxidation** by the liver. Cortisol also facilitates action of other hyperglycemic hormones (permissive effects) such as glucagon, epinephrine, and growth hormone, which further inhibit uptake and utilization of glucose by the peripheral tissues.

Growth Hormone

Anterior pituitary secretes growth hormone. Its secretion is stimulated under the influence of hypoglycemia. Prolonged administration of the growth hormone **stimulates secretion of insulin**. As a result of increased insulin secretion, β-cells are exhausted producing diabetes like situation. Growth hormone also **decreases glucose uptake** and **utilization by muscle**. It also **promotes lipolysis in the adipose tissue**.

Thyroxine

Thyroxine is secreted by the **thyroid gland**. It **stimulates intestinal absorption of glucose** and promotes **glycogenolysis** as well as **gluconeogenesis** in the liver. Hyperthyroidism is, generally, associated with hyperglycemia and mild diabetes.

METABOLIC DISORDERS

DIABETES MELLITUS

Diabetes mellitus is a major disorder of carbohydrate metabolism. It is characterized by persistent **hyperglycemia** with or without glycosuria (**glucosuria**). This is the commonest endocrine disease caused by the **deficiency in secretion** or **action of insulin**. Besides insulin, several diabetogenic hormones influence metabolism of glucose.

Diabetes mellitus is defined as a state of **chronic hyperglycemia**, which may be a result of genetic and/or environmental factors. It is a multifactorial disease. There are two major clinical classes of diabetes mellitus which are referred to as insulin dependent diabetes mellitus (IDDM) and noninsulin dependent diabetes mellitus (NIDDM).

Insulin Dependent Diabetes Mellitus

Insulin dependent diabetes mellitus (IDDM) or **type I diabetes** is also called **juvenile-onset diabetes** because it usually appears in the childhood or in **younger age group** (commonly less than 40 years of age). There is absolute deficiency of insulin, due to a gradual **depletion of β-cells** of the pancreas which may get destroyed by an autoimmune process.

- IDDM is characterized by **hyperglycemia**, **hyperlipoproteinemia** (raised chylomicron and VLDL) and severe **ketoacidosis**. This, in turn, suggests that besides defects in carbohydrate metabolism there are abnormalities in fat and protein metabolism also in such patients.
- **Hyperglycemia** in IDDM is a result of inability of the insulin-dependent tissues to take up glucose as well as due to accelerated hepatic **gluconeogenesis** from amino acids (derived from muscle protein).
- **Increased lipolysis** (in the adipose tissue) and accelerated **fatty acid oxidation** (in the liver) result in **ketoacidosis**.
- **Insulin deficiency** also reduces lipoprotein lipase activity, thereby resulting in **hyperchylomicronemia**.
- Patients with IDDM can usually be recognized by abrupt appearance of **polyuria** (frequent urination), **polydypsia** (excessive thirst) and **polyphagia** (excessive hunger), often triggered by the stress or some other illness. These symptoms are usually accompanied with fatigue, weight loss and weakness.
- Its diagnosis is confirmed by high fasting blood glucose, commonly accompanied with ketoacidosis. **Insulin injection**, though does not cure the disease but promotes glucose uptake by the tissues and inhibits gluconeogenesis, lipolysis and proteolysis.
- **Life span** of the patient is **reduced** as a result of the degenerative complications such as kidney malfunction, nerve impairment and cardiovascular disease.
- Hyperglycemia also leads to **blindness** through retinal degeneration and glycosylation of the lens proteins, which, in turn, causes **cataract**.

Noninsulin Dependent Diabetes Mellitus

Majority of the diabetic patients (over 80%) suffer from noninsulin dependent diabetes mellitus (NIDDM) or **type II diabetes**. It is also called **maturity-onset diabetes** since it usually occurs in the middle age group (usually more than 40 years of age), in those who are, generally, obese. Occurrence of the disease is almost completely determined by the genetic factors. NIDDM develops gradually, without obvious symptoms. Metabolic alterations are milder than IDDM.

- NIDDM is characterized by **hyperglycemia**, often with **hypertriglyceridemia.** In spite of high level of insulin, glucose levels are poorly controlled because of the lack of normal response to insulin.
- **Insulin resistance** in these patients may be due to increased expression of the tumor necrosis factor-α in the adipocytes of obese individuals. Hyperglycemia is, mainly, a result of the **poor peripheral utilization of glucose**, especially in muscle.
- Ketoacidosis does not develop because adipocytes remain sensitive to the effect of insulin.
- Rapid *de novo* synthesis of fatty acids and VLDL leads to **hypertriglyceridemia** without hyperchylomicronemia.
- Blood glucose concentration is much higher than normal, particularly after a meal.
- Weight reduction and dietary modifications often correct hyperglycemia of type II diabetes. **Hypoglycemic agents**, such as **sulphonylureas**, may be required to achieve a satisfactory fall in blood glucose level.

Salient features of diabetes mellitus are shown in Table 29.4.

For any form of diabetes, metabolic derangement is due to elevated glucagon/insulin ratio. In addition, there may be weight loss in IDDM. Long-term complications of the disease include acute myocardial infarction (diabetic patients often present with painless acute myocardial infarction, i.e. silent MI), due to accelerated atherosclerosis and coronary heart disease, nephropathy, retinopathy, neuropathy, infections and delayed wound healing.

Table 29.4 Salient features of the two types (IDDM and NIDDM) of diabetes mellitus

	IDDM	NIDDM
Type of diabetes	Juvenile onset (Type I)	Maturity-onset (Type II)
Prevalence	About 20%	About 80%
Age of onset	< 40 years	> 40 years
Body habitus	Normal to wasted (weight loss)	Obese
Plasma insulin	Low (due to β-cell destruction)	Normal to high (insulin resistance)
Plasma glucagon	High (suppressible)	High (resistant)
Acute complications	Ketoacidosis	Hyperosmolar coma (ketoacidosis is rare)
Insulin therapy	Responsive	Resistant
Oral hypoglycemic agents	Unresponsive	Responsive

Important Questions

1. Outline reactions of tricarboxylic acid (TCA) cycle. Give bioenergetics of the cycle and explain its biomedical importance.
2. Outline hexose monophosphate (HMP) shunt reactions. Discuss biomedical importance of HMP shunt.
3. Outline glycolysis. What are its regulatory steps? Give its bioenergetics.
4. Define glycogenolysis. Outline glycogenolysis.
5. What is glycogenesis? Outline glycogenesis.
6. Write notes on:
   i. Hormonal regulation of blood glucose
   ii. Beriberi
   iii. Diabetes mellitus
   iv. Cobamide coenzymes
   v. Glycogen storage diseases

Chapter

30

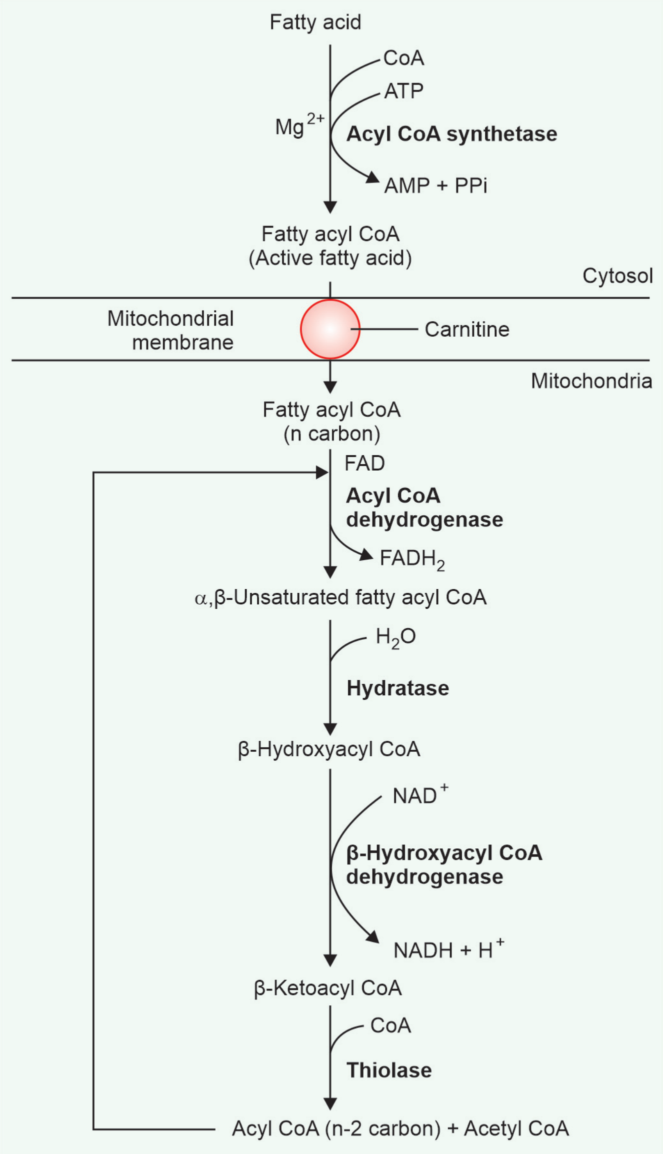
Metabolism of Lipids

Digestion of dietary lipids results in the formation and release of chylomicrons, which transport triacylglycerols to liver and adipose tissue. Triacylglycerols are subsequently hydrolyzed to release fatty acids which are oxidized to produce energy.

METABOLISM OF FATTY ACIDS

Major pathway for the oxidation of fatty acids occurs in the mitochondria. This pathway is called **β-oxidation of fatty acids** and was proposed by **Knoop**.

In this process, **oxidation** of fatty acids **occurs at the β-carbon atom** (carbon-3) and two-carbon fragments are successively removed from the carboxyl end of fatty acyl CoA. This, in turn, results in the **elimination of two terminal carbon atoms as acetyl CoA**, thereby leaving fatty acyl CoA that has two carbons less than the original fatty acid. The process of β-oxidation of a fatty acid is outlined in Fig. 30.1.



Fatty acid
CoA
ATP
Mg^{2+}
Acyl CoA synthetase
AMP + PPi
Fatty acyl CoA
(Active fatty acid)
Cytosol
Mitochondrial membrane
Carnitine
Mitochondria
Fatty acyl CoA
(n carbon)
FAD
Acyl CoA dehydrogenase
$FADH_2$
α,β-Unsaturated fatty acyl CoA
H_2O
Hydratase
β-Hydroxyacyl CoA
NAD^+
β-Hydroxyacyl CoA dehydrogenase
$NADH + H^+$
β-Ketoacyl CoA
CoA
Thiolase
Acyl CoA (n-2 carbon) + Acetyl CoA

Fig. 30.1: Outline of β-oxidation of fatty acid

Outline of β-Oxidation of Fatty Acids

β-Oxidation of a fatty acid involves **activation of fatty acid**, its **transport into the mitochondria** and **oxidation** within the mitochondria.

Activation of Fatty Acids

A long chain fatty acid (fatty acid containing > 12 carbons) enters a cell where it is converted to its CoA derivative by the enzyme long chain fatty acyl CoA synthetase (thiokinase) in the cytosol.

Transport of Fatty Acids from Cytoplasm into Mitochondria

The process of **β-oxidation** occurs in the mitochondrial matrix, thus, fatty acid must be transported across the mitochondrial membrane. Short chain fatty acids can cross the inner mitochondrial membrane and are activated to their CoA derivatives within the mitochondria. However, it is impermeable to a long chain fatty acid. A long chain fatty acid is transported from cytosol to the mitochondrial matrix with the help of a specialized carrier called **carnitine**. This process is called carnitine shuttle (Fig. 30.2).

- In the process, an acyl group is first transferred from the cytosolic CoA to carnitine, by the enzyme carnitine palmitoyl transferase-I (CPT-I). This enzyme is associated with the outer mitochondrial membrane and is also referred as carnitine acyl transferase-I (CAT-I). This reaction forms acylcarnitine and releases CoA.
- Thereafter, acyl carnitine is transported into the mitochondrial matrix by a protein called carnitine acyl-translocase.
- Finally, CPT-II (CAT-II) which is present in the inner mitochondrial membrane, catalyzes transfer of the acyl group from acylcarnitine to CoA into the mitochondrial matrix and releases carnitine.

Carnitine is obtained from dietary meat. A small amount of it can also be synthesized in the liver and kidney from lysine and methionine. **Carnitine deficiency** can be observed in patients with a liver disease, malnutrition or in strict vegetarians.

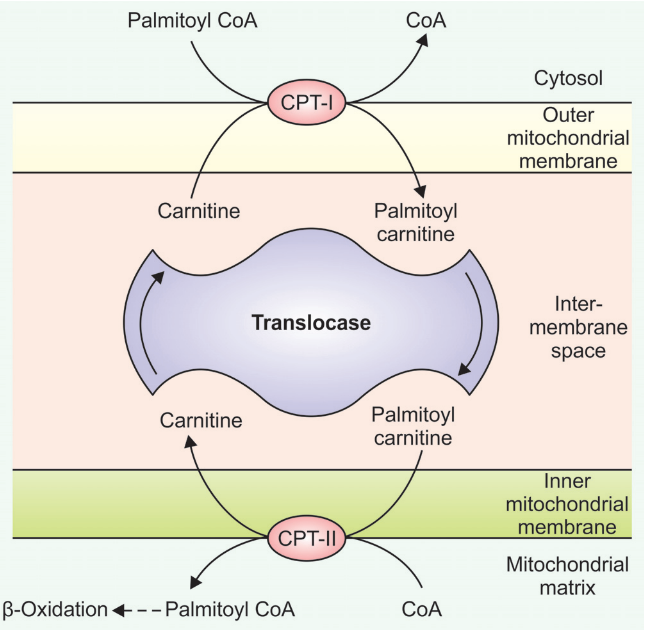


Fig. 30.2: Role of carnitine in the transport of fatty acid from cytosol to mitochondria

β-Oxidation of Fatty Acids in Mitochondria

The process of β-oxidation consists of four reactions, which result in shortening of the fatty acid chain by two carbons. It includes:

- **Oxidation:** After activation of the fatty acid, such as palmitic acid, and its entry into the mitochondrial matrix, flavoprotein containing **acyl CoA dehydrogenase removes two hydrogens** from the α- and β-positions of the fatty acyl CoA and **produces $FADH_2$**. The fatty acyl CoA is changed to a,b-unsaturated fatty acyl CoA (enoyl CoA).
- **Hydration:** In the next step, **enoyl CoA hydratase adds a molecule of water** at the double bond and converts the enoyl CoA to β-hydroxyacyl CoA.
- **Oxidation:** Thereafter, **β-hydroxyacyl CoA dehydrogenase oxidizes β-hydroxyacyl CoA** to β-ketoacyl CoA.
- **Cleavage:** In the last step, acyl CoA:acyltransferase, also called **thiolase**, in the presence of CoA, results in thiolytic **cleavage of the β-ketoacyl CoA** into acetyl CoA and fatty acyl CoA which has two carbons less than the original fatty acyl CoA.

The above four steps are repeated, i.e. the fatty acyl CoA again undergoes above four set of reactions, starting with acyl CoA dehydrogenase. Each cycle produces acetyl CoA, $FADH_2$ and NADH. Thus, the fatty acid is sequentially degraded to acetyl CoA.

ATP Production During β-Oxidation

When a molecule of **palmitic acid** (**$C_{15}H_{31}COOH$**) undergoes β-oxidation, it **releases 8 molecules of acetyl CoA** in seven rounds. In each round, one molecule each of $FADH_2$ and $NADH+H^+$ are also produced, which, in turn, generate 1.5 and 2.5 ATP, respectively.

Thus, a total of 28 ATP are produced during this process. In addition, each molecule of acetyl CoA, when oxidized via the citric acid cycle, produces 10 ATP. Therefore, additionally, 80 ATP are produced from 8 molecules of acetyl CoA. Hence, a total of **108 ATP** are produced from one molecule of palmitic acid.

As two high-energy phosphate bonds are hydrolyzed from one molecule of ATP, which is changed to AMP, thus, a total of 2 ATP are used during activation of the fatty acid.

Therefore, there is a **net yield of 106 ATP** when one molecule of palmitic acid (fatty acid with 16 carbons) is completely oxidized.

β-Oxidation of a Fatty Acid Containing Odd Number of Carbon Atoms

β-Oxidation of a saturated fatty acid with odd number of carbon atoms proceeds in the same way as a saturated fatty acid with even number of carbons, until final three carbons are reached. Thus, **in addition to acetyl CoA**, a fatty acid with odd number of carbon atoms **also produces** a molecule of **propionyl CoA** (containing 3 carbons), which is converted to succinyl CoA and is used in the TCA cycle.

β-Oxidation of Unsaturated Fatty Acids

Unsaturated fatty acids are also activated, transported across the inner mitochondrial membrane and undergo β-oxidation, by the process similar to that of a saturated fatty acid. However, since the double bonds in the naturally occurring unsaturated fatty acids are found in cis-configuration (compared to those produced during β-oxidation, which have trans-configuration) hence, **three additional enzymes are required** for their oxidation. These include:

- **Enoyl CoA isomerase**,
- **Dienoyl CoA reductase** and
- **3,2-Enoyl CoA isomerase**

Thus, in addition to the other enzymes of β-oxidation, these three enzymes are also required for the oxidation of the unsaturated fatty acids.

Inborn Error of β-Oxidation of Fatty Acids

Sudden infant death syndrome

Sudden infant death syndrome (**SIDS**), also called **Reye's syndrome**, is due to the deficiency of **medium chain fatty acyl CoA dehydrogenase** enzyme. It is an autosomal recessive disorder, which, in turn, reduces fatty acid oxidation. Features of the disease include vomiting, lethargy and coma, accompanied with the hypoketotic hypoglycemia and dicarboxylic aciduria. Symptoms appear after more than 12 hours of fast and are more complicated on prolonged starvation, usually within two years of life. Treatment includes a carbohydrate-rich diet.

METABOLIC FATES OF ACETYL COA

Acetyl CoA is mainly **used** in the **Krebs cycle**. Besides **Krebs cycle**, acetyl CoA can also be used for the **synthesis of fatty acids** and **cholesterol**. A small quantity of it is also converted to **ketone bodies** in the liver (Fig. 30.3).

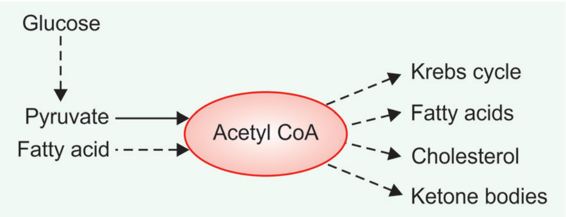


Fig. 30.3: Production and utilization of acetyl CoA

SYNTHESIS OF FATTY ACIDS

Mammals can synthesize a major portion of the saturated as well as monounsaturated fatty acids. First, a saturated, straight chain, 16-carbon-containing fatty acid, i.e. palmitic acid, is synthesized, which is subsequently changed to other fatty acids. The process of fatty acid synthesis is called ***de novo* synthesis**.

In human beings, fatty acid synthesis occurs, primarily, in the cytosol, in the liver, lactating mammary glands and to a lesser extent in the adipose tissue.

The process of fatty acid synthesis includes the transport of acetyl CoA to cytosol, its conversion to malonyl CoA and synthesis of palmitic acid (16-carbon fatty acid).

Transport of Acetyl CoA from Mitochondria to Cytosol

Acetyl CoA, which is **produced in the mitochondria**, first comes out of the mitochondria to the cytosol, where fatty acid synthesis occurs. Mitochondrial membrane, however, is impermeable to CoA. Thus, first step in fatty acid synthesis is the transfer of the acetate units, from mitochondria to the cytosol. For this purpose:

- **Acetyl CoA condenses with oxaloacetate and forms citrate**.
- **Citrate is then translocated from mitochondria to the cytosol**, where it is cleaved **by the enzyme ATP-citrate lyase and forms acetyl CoA and oxaloacetate, in the cytosol** (Fig. 30.4).

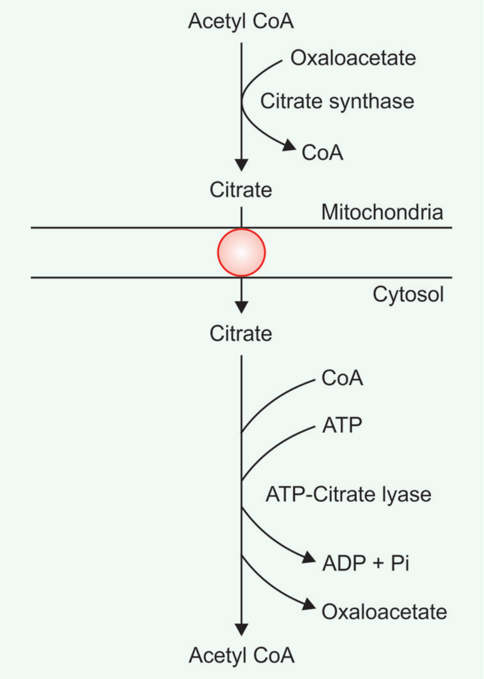


Fig. 30.4: Transport of acetyl CoA from mitochondria to cytosol

Conversion of Acetyl CoA to Malonyl CoA

In the cytosol, **acetyl CoA** is carboxylated **to malonyl CoA** by a biotin-containing enzyme, referred to as **acetyl CoA carboxylase**. This coenzyme is covalently linked to the lysyl residue of the enzyme, which requires ATP and CO_2 (Fig. 30.5).

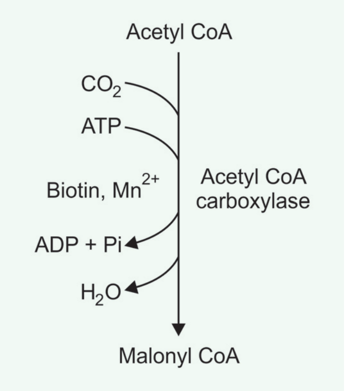

Acetyl CoA
CO_2
ATP
Biotin, Mn^{2+}
Acetyl CoA carboxylase
ADP + Pi
H_2O
Malonyl CoA

Fig. 30.5: Conversion of acetyl CoA to malonyl CoA

Synthesis of Palmitic Acid

In eukaryotes, synthesis of palmitic acid occurs in the cytosol by the enzyme referred to as fatty acid synthase.

Fatty Acid Synthase

It is a multifunctional, dimeric enzyme, composed of the two identical subunits. Its each monomer is a multicatalytic polypeptide with seven different enzyme activities plus a domain that covalently binds a molecule of 4′-phosphopantotheine. During fatty acid synthesis, 4′-phosphopantotheine carries acetyl and acyl units on its terminal thiol (–SH) groups. Individually, different enzyme activities of the fatty acid synthase complex are designated as:

- **Acetyl CoA-ACP acetyl transacylase**,
- **Malonyl CoA-ACP transacylase**,
- **Condensing enzyme** (β-Ketoacyl-ACP synthase),
- β-Ketoacyl ACP **reductase**,
- β-Hydroxyacyl ACP **dehydratase** and
- Enoyl-ACP **reductase**.

These enzyme activities are separate catalytic domains that are present on each subunit of the multicatalytic fatty acid synthase complex. Fatty acid synthase contains **two –SH groups**, called pantotheinyl-SH group, which is referred to as the **ACP-SH**, and the thiol-SH group that is designated as the **CYS-SH**.

In prokaryotes, fatty acid synthase is a multienzyme complex and the 4′-phosphopantotheine domain is present on a separate protein, referred to as acyl carrier protein (ACP).

Outline of Fatty Acid Synthesis

Outline of fatty acid synthesis is given in Fig. 30.6.

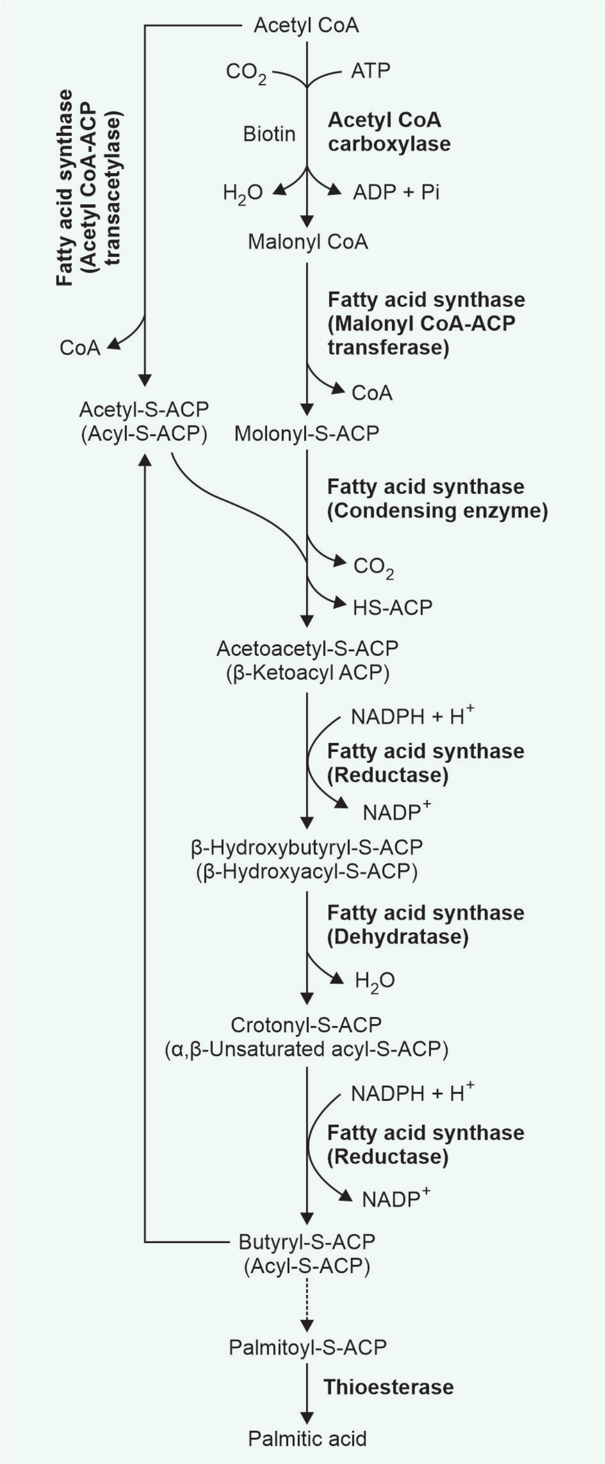

Acetyl CoA
CO_2
ATP
Biotin
Acetyl CoA carboxylase
H_2O
ADP + Pi
Malonyl CoA
Fatty acid synthase (Acetyl CoA-ACP transacetylase)
CoA
Fatty acid synthase (Malonyl CoA-ACP transferase)
CoA
Acetyl-S-ACP (Acyl-S-ACP)
Molonyl-S-ACP
Fatty acid synthase (Condensing enzyme)
CO_2
HS-ACP
Acetoacetyl-S-ACP (β-Ketoacyl ACP)
NADPH + H^+
Fatty acid synthase (Reductase)
$NADP^+$
β-Hydroxybutyryl-S-ACP (β-Hydroxyacyl-S-ACP)
Fatty acid synthase (Dehydratase)
H_2O
Crotonyl-S-ACP (α,β-Unsaturated acyl-S-ACP)
NADPH + H^+
Fatty acid synthase (Reductase)
$NADP^+$
Butyryl-S-ACP (Acyl-S-ACP)
Palmitoyl-S-ACP
Thioesterase
Palmitic acid

Fig. 30.6: Outline of fatty acid synthesis

1. Fatty acid synthesis starts with the **transfer of the molecule of acetate, from acetyl CoA to the ACP-SH**. This reaction is catalyzed by the **acetyl CoA-**

ACP acetyl transacylase domain of the fatty acid synthase complex and the two-carbon fragment is transferred to the **CYS-SH,** which is a temporary holding site.

At the same time, **malonyl CoA-ACP transacylase** domain of the fatty acid synthase **transfers three-carbon malonate unit from malonyl CoA** to the vacant **–SH** group of ACP.

2. In the next step, malonyl group loses CO_2 and transfers the acetyl group, by β-ketoacyl-ACP-synthase domain of the fatty acid synthase, which, in turn, causes **condensation of the 2 acetyl group on the CYS–SH** and forms a **four-carbon acetoacetyl unit that is attached to the ACP domain**.
3. **Thereafter,** β-ketoacyl ACP **reductase** domain of the fatty acid synthase reduces keto group of the **acetoacetyl-S-ACP to β-hydroxybutyryl-S-ACP** (β-hydroxyacyl-S-ACP).
4. In the next step, β-hydroxyacyl-ACP **dehydratase domain** of the fatty acid synthase removes a molecule of H_2O from **β-hydroxyacyl-S-ACP** and introduces a double bond in it, to form crotonyl-S-ACP (**α,β-transbutenyl-ACP** or α,β-unsaturated acyl-S-ACP).
5. **In the next step, crotonyl-S-ACP is reduced by the enoyl-ACP reductase domain** of the fatty acid synthase, and is converted **to butyryl-S-ACP** (acyl-S-ACP).

   As a result of these reactions, a four-carbon compound is formed whose three terminal carbons are fully saturated and remain attached to ACP. These steps are again repeated, beginning with the transfer of the butyryl-chain to the cys-SH. This cycle of the reactions is repeated five more times, each time incorporating two-carbon units, which are derived from malonyl CoA, and are attached to the growing fatty acid chain, at the carboxyl end. After the chain length reaches 16 carbons, process is terminated with the formation of palmitoyl-S-ACP.
6. Thereafter, **palmitoyl thioesterase cleaves the thioester bond with the release of palmitate.**

   Reducing equivalents (NADPH + H^+), which are used in the biosynthesis of fatty acids, are derived from the pentose phosphate pathway.

Synthesis of Other Fatty Acids

Palmitate, produced by fatty acid synthase catalyzed reactions is modified by chain elongation and desaturation.

METABOLISM OF CHOLESTEROL

An adult man contains about 1 g cholesterol/kg body weight. Cholesterol **content in normal human blood** varies from 130 to 220 mg/100 mL, being equally distributed between plasma and erythrocytes. About 30% of the circulating cholesterol occurs **free**, while remaining of it exists in the form of **cholesterol esters**. Usually, a long chain fatty acid is attached to the –OH group (by an ester linkage) on carbon 3 of the ring A of cholesterol.

SYNTHESIS OF CHOLESTEROL

Important sites for cholesterol biosynthesis include liver, skin, intestine, adrenal cortex and reproductive tissues, including ovaries, testes and placenta. All the carbon atoms of **cholesterol** are derived **from acetate** (acetyl CoA), which is obtained from several sources, such as oxidation of long chain fatty acids, ketogenic amino acids and glucose (via pyruvate). Synthesis of cholesterol is called *de novo* synthesis.

Outline of Cholesterol Synthesis

De novo synthesis of cholesterol is outlined in Fig. 30.7.

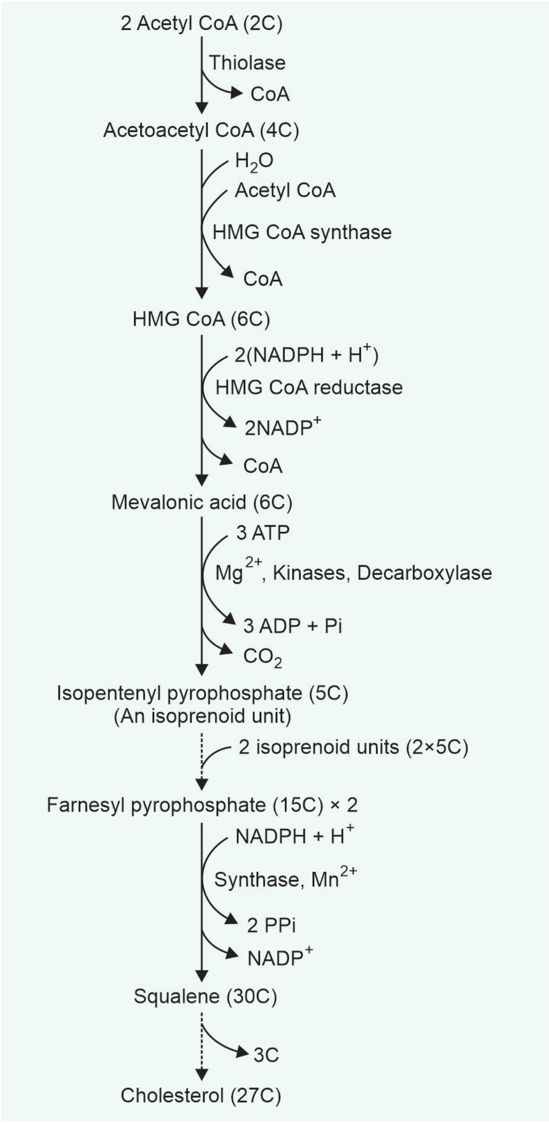

2 Acetyl CoA (2C)
Thiolase
CoA
Acetoacetyl CoA (4C)
H2O
Acetyl CoA
HMG CoA synthase
CoA
HMG CoA (6C)
2(NADPH + H+)
HMG CoA reductase
2NADP+
CoA
Mevalonic acid (6C)
3 ATP
Mg2+, Kinases, Decarboxylase
3 ADP + Pi
CO2
Isopentenyl pyrophosphate (5C)
(An isoprenoid unit)
2 isoprenoid units (2×5C)
Farnesyl pyrophosphate (15C) × 2
NADPH + H+
Synthase, Mn2+
2 PPi
NADP+
Squalene (30C)
3C
Cholesterol (27C)

Fig. 30.7: Outline of cholesterol synthesis

- First, **two molecules of acetyl CoA** condense to **form acetoacetyl CoA**. This reaction is catalyzed by the enzyme **acetoacetyl CoA thiolase**.
- Thereafter, in the presence of the enzyme β-hydroxy-β-methylglutaryl CoA synthase (**HMG CoA synthase**), **acetoacetyl CoA** further **condenses with** another molecule of **acetyl CoA** and **forms** β-hydroxy-β-methylglutaryl CoA (**HMG CoA**).
- In the next step, an NADPH-dependent enzyme, **HMG CoA reductase**, converts HMG CoA to **mevalonic acid**. This is the **regulatory step** in cholesterol biosynthesis.
- Thereafter, there occur stepwise transfer of the two γ-phosphate groups from two molecules of ATP. These reactions are catalyzed by two kinases, called **mevalonate kinase** (enzyme I) and **phosphomevalonate kinase** (enzyme II).
- Subsequently, decarboxylation takes place by the enzyme decarboxylase (pyrophosphomevalonate decarboxylase). Thus, phosphorylation and decarboxylation of **mevalonic acid forms isopentenyl pyrophosphate**, which is also called **active isoprenoid unit**.
- In the next step, the isoprenoid unit (isopentenylpyrophosphate) is isomerised to another isoprenoid unit, designated as 3,3-dimethylallylpyrophosphate, by the enzyme isopentenylpyrophosphate isomerase.
- Thereafter, **stepwise condensation of the three isoprenoid units (5C units)** leads to the formation of a 15-carbon unit called **farnesylpyrophosphate** (15C).
- In this process, first, a molecule each of isopentenylpyrophosphate and 3,3-dimethylallylpyrophosphate condense together and forms a 10-carbon unit, called geranylpyrophosphate (10C). It further condenses with another molecule of isopentenylpyrophosphate and forms farnesylpyrophosphate (15C). This reaction is catalyzed by the enzyme geranyltransferase.
- In the next step, fusion of the **two molecules of farnesylpyrophosphate forms a 30-carbon compound called squalene** (30C).
- Subsequently, by ring closure and removal of the three methyl groups, **squalene is converted to cholesterol**, which has 27 carbons.

CATABOLISM OF CHOLESTEROL

Free cholesterol is present in abundance, in **bile**. It is also present in large amount in the **myelin structure of the brain**. Cholesterol also occurs in the esterified form in the **cell membrane**. **Besides**, cholesterol is also a precursor of the **bile acids**, **steroid hormones** and **vitamin D** (Fig. 30.8).

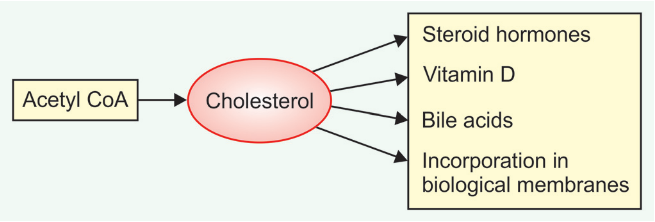


Fig. 30.8: Precursor for *de novo* synthesis of cholesterol and its metabolic fates

Regulation of Cholesterol Metabolism

Cholesterol synthesis is regulated by dietary cholesterol, feedback inhibition and its disposal by the liver.

- **Dietary cholesterol:** Rate of *de novo* synthesis of cholesterol is inversely related to the amount of dietary cholesterol. When dietary cholesterol intake is reduced, cholesterol synthesis is increased in the liver and the intestine, to meet needs of the other tissues. On the other hand, when dietary cholesterol intake is increased, its synthesis is reduced.
- **Feedback inhibition:** Cholesterol inhibits its own synthesis by feedback inhibition. HMG CoA reductase regulates cholesterol synthesis. Cholesterol inhibits the activity of HMG CoA reductase by suppressing its synthesis and promoting inactivation.
- **Disposal by the liver:** Liver removes cholesterol by several processes:
  - **Esterification:** Lecithin:cholesterol acyltransferase (LCAT) and HDL are important for the esterification of cholesterol.

    LCAT is a plasma enzyme which is produced, mainly, by the liver. It transfers one fatty acid from carbon-2 of lecithin to the 3-hydroxyl group of cholesterol. This reaction is freely reversible and utilizes cholesterol, which is present in **HDL**.

    Cholesterol esters, so formed, diffuse into core of the HDL particle, where they are transported from the tissues and plasma to the liver, which further metabolizes and excretes it. This is referred to as the **reverse transport of cholesterol** since HDL provides the vehicle for the transport of cholesterol from the peripheral tissues to the liver.

    In a normal healthy adult, on a low cholesterol diet, about 1300 mg of cholesterol is returned to the liver, each day.
  - **Excretion:** Free cholesterol as well as bile acids/salts are excreted in the bile.
  - **Incorporation into VLDL:** In the liver, cholesterol is also incorporated into VLDL and is secreted into circulation.

ATHEROSCLEROSIS

Atherosclerosis (arteriosclerosis) is a disease of arterial wall. It is characterized by the accumulation of **cholesterol** and its esters in cells which are derived from monocyte-macrophage that line smooth muscle cells. This, in turn, results in the formation of **atherosclerotic plaque**, **which narrows the blood vessels** and serves as the site of thrombus formation.

High calorie diet, or diet rich in saturated fat and cholesterol, hypercholesterolemia, sedentary life style, stress and strain, obesity, hypertension and smoking are important contributory factors, favorable in the development of atherosclerosis.

Risk of developing the disease is directly related to the concentration of the LDL-cholesterol, in plasma. It is also inversely related to the plasma concentration of HDL-cholesterol. Therefore, **LDL-cholesterol is called bad cholesterol** while **HDL-cholesterol** is referred to as **good cholesterol**.

Drugs like **cholestyramine** bind bile salts and promote their excretion in stool. This, in turn, **increases the rate of bile salt synthesis** and LDL uptake by the liver. **Lovastatin**, the drug that is used in the treatment of **hyperlipidemia**, is an **inhibitor of the enzyme HMG CoA reductase**. Hence, it decreases endogenous synthesis of cholesterol and stimulates LDL uptake (via LDL receptors) by the tissues.

Dietary Recommendations

- Reduce the total amount of fat. Not more than 30% of total energy intake should come from fat.
- Reduce the use of animal fat. Not more than one-third of the total fat (less than 10% calories/day) should come from saturated fat, with the remainder from unsaturated fat.
- Reduce the intake of cholesterol. Dietary cholesterol should be less than 300 mg/day.
- Trans-fatty acid intake should limit to less than 1% calories/day.
- Increase omega-3 fatty acid intake from dietary or supplementary fish oil.
- Eat five or more servings of fruits and vegetables/ day.
- Reduce salt intake.

Other Lifestyle Interventions

In addition to dietary advice, other lifestyle interventions include:

- Promotion of physical activity, e.g. adults should perform atleast 30 minutes of moderate activity (brisk walking), atleast 5 days per week. Maintaining ideal body weight emphasizes activity.
- Limit sedentary activities and give up smoking.

OUTLINE OF LIPOPROTEINS METABOLISM

Metabolism of lipoproteins is outlined in Fig. 30.9.

Metabolism of Chylomicrons

Chylomicrons are triglyceride-rich particles that are assembled in the intestinal mucosal cells. They carry dietary triacylglycerols, cholesterol and cholesteryl esters to peripheral (extrahepatic) tissues.

- **Intestinal mucosal cells release nascent chylomicrons** which contain apo B 48 that is synthesized in the intestine.
- Nascent chylomicrons **receive apo E and apo C from** the circulating HDL. Apo CII, in turn, activates lipoprotein lipase and hydrolyzes triacylglycerols to monoacylglycerols, free fatty acids and glycerol. As a result of **hydrolysis of triacylglycerols**, which is the major component of chylomicrons, chylomicrons decrease in size and increase in density.
- Thereafter, **apo C is returned to HDL** and the **leftover particles are called chylomicron remnants**.
- Chylomicron remnants bind to apo E receptors on hepatocytes. They are then internalized and their lipids, including cholesteryl esters, are either utilized or stored, intracellularly. A small amount of the released free fatty acids enters the circulation and contributes to plasma free fatty acid pool.

Metabolism of very Low Density Lipoproteins

Very low density lipoproteins (VLDL), also called pre b-lipoproteins, are composed of triacylglycerols, like chylomicrons, but their function is to transport lipid from liver to the peripheral tissues.

- VLDL is **released from the liver as nascent VLDL**, similar to the release of nascent chylomicrons from the intestinal mucosa. Nascent VLDL particles contain apo B 100 and apo A1.
- Subsequently, they **acquire apo CII and apo E from the circulating HDL**.
- During circulation, **lipoprotein lipase hydrolyses triacylglycerols and releases free fatty acids**, which are taken-up by the peripheral tissues.
- **Apo C and apo E are transferred to HDL**.
- In addition, cholesteryl esters, which are synthesized in the plasma as a result of the action of lecithin cholesterol acyltransferase (LCAT), are transferred from HDL to VLDL and IDL via cholesteryl ester transfer protein (CETP). As a result of it, their particle

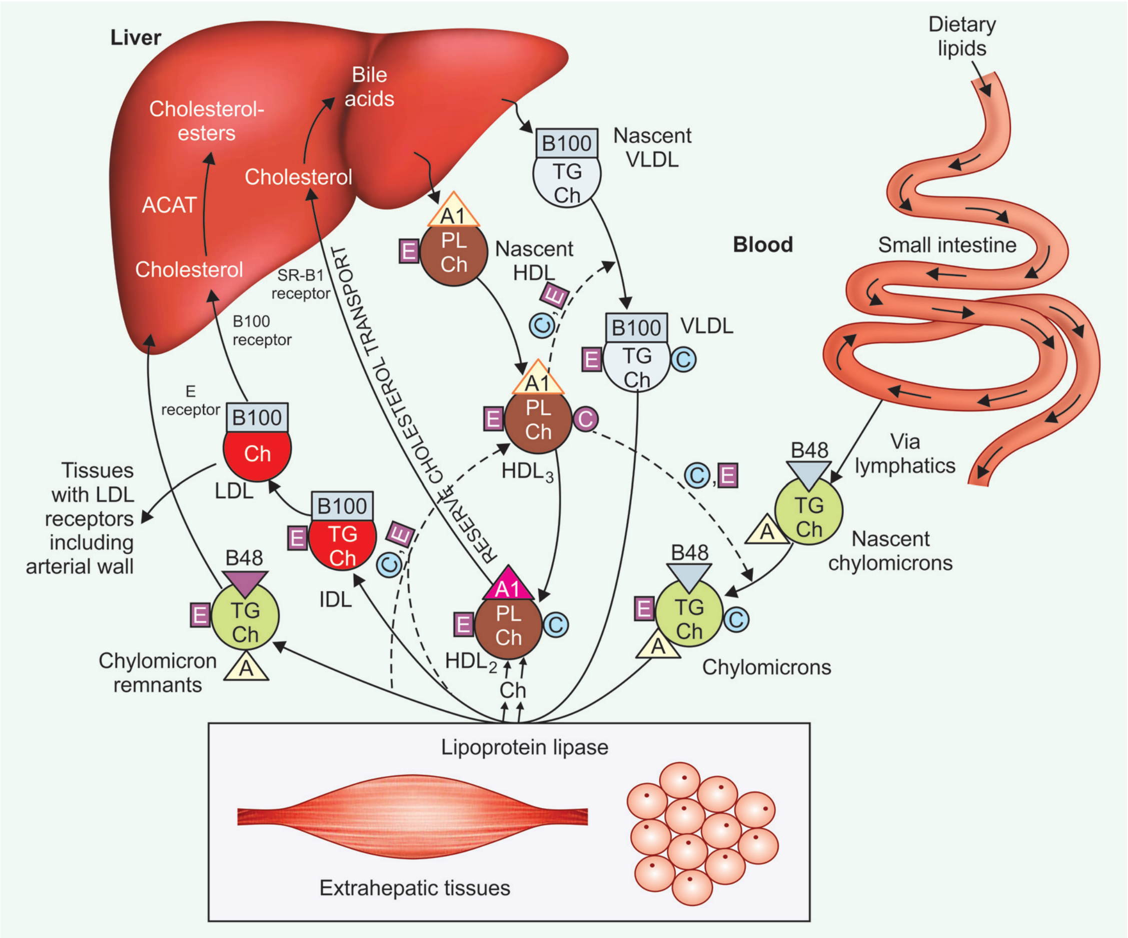

Liver
Cholesterol-esters
ACAT
Cholesterol
Bile acids
Cholesterol
SR-B1 receptor
B100 receptor
E receptor
B100
Ch
LDL
Tissues with LDL receptors including arterial wall
B100
TG
Ch
E
IDL
B48
TG
Ch
E
A
Chylomicron remnants
REVERSE CHOLESTEROL TRANSPORT
B100
TG
Ch
Nascent VLDL
A1
PL
Ch
E
Nascent HDL
E
C
A1
PL
Ch
E
C
HDL3
A1
PL
Ch
E
C
HDL2
Ch
B100
TG
Ch
E
C
VLDL
Blood
C, E
Dietary lipids
Small intestine
Via lymphatics
B48
TG
Ch
A
Nascent chylomicrons
B48
TG
Ch
E
C
A
Chylomicrons
Lipoprotein lipase
Extrahepatic tissues

Fig. 30.9: Outline of lipoproteins metabolism

size is reduced and density is increased. **VLDL particles** are, hence, **converted into VLDL remnants**, which are also designated as **intermediate density lipoproteins** (**IDL**). IDL particles are removed by the liver or converted to the LDL.

Metabolism of Low Density Lipoproteins

Low density lipoproteins (LDL) contain high concentration of cholesterol. They retain apo B 100 and lose other apoproteins to HDL. Primary function of LDL is to transport cholesterol from liver to the extrahepatic tissues.

- **LDL binds to its receptors**, which are present in the cell membrane. The LDL receptors are negatively charged glycoproteins that are clustered in the pit on the cell membrane and recognize apo B 100, which, in turn, acts as a ligand between the IDL and its receptor. The intracellular side of the pits is coated with a protein, called clathrin, which stabilizes shape of the pit. Ability of the tissues to take up LDL depends upon the rate of transcapillary transport as well as the number of receptors. The number of these receptors is regulated by cholesterol content of the cell.
- After binding, LDL particles are **internalized by endocytosis**, in the form of vesicles. These **vesicles** rapidly loose clathrin coat, **fuse with the other vesicles** and **form endosomes** (large vesicles). This, in turn, results in **separation of LDL particles from their receptors**.
- **Receptors are recycled,** whereas **endosome contents are degraded**, resulting in **release of cholesterol**, free fatty acids, phospholipids and amino acids. Free cholesterol is used for membrane biosynthesis or converted to steroid hormones. Excess of the cholesterol, however, is reesterified by acylcholesterol acyltransferase (ACAT) and is stored.

In man, 70–80% of LDL is removed by LDL receptors while rest of it is removed by the nonspecific endocytosis via the scavenger pathway. The latter is also a receptor-mediated process involving phagocytic cells in the reticuloendothelial system.

Metabolism of High Density Lipoproteins

High density lipoprotein (HDL) particles are synthesized in the liver and are **released into the blood stream** by exocytosis.

- Secretory HDL particles are called **nascent HDL** and are discoid in shape. Nascent HDL is composed of apo A, apo E, phospholipids and free cholesterol.
- There also occurs **transfer of apo C** from the chylomicrons and VLDL **to nascent HDL**, which is converted to **mature HDL**, also called HDL_3.
- From HDL_3, cholesteryl esters (produced by the action of LCAT) are shifted to VLDL and IDL. At the same time, HDL_3 acquires triacylglycerols and is converted to **LDL**.

KETOSIS

Clinical condition resulting from the **increased synthesis of ketone bodies** is called **ketosis**. Ketosis occurs **in severe diabetes**, **starvation** or **on high-fat low-carbohydrate diet**.

- Three compounds, **acetoacetate**, **β-hydroxybutyrate** and **acetone**, are collectively referred to as **ketone bodies** (acetone bodies).
- The process of the **synthesis of ketone bodies** is called **ketogenesis**.
- Ketone bodies are diffused into the blood. Their **increased** concentration in blood is called **ketonemia**.
- In a normal man, concentration of the ketone bodies is usually less than 3 mg/100 mL of blood. In conditions, such as during prolonged starvation or impaired glucose oxidation, like diabetes mellitus, when fat becomes the source of energy and lipolysis is accelerated, there is increased production of acetyl CoA, which cannot be utilized in the Krebs cycle (due to lack of oxaloacetate) and forms ketone bodies.
- Ketonemia results in **increased excretion of ketone bodies in urine**. It is referred to as **ketonuria**.
- The process of degradation (oxidation) of ketone bodies is called **ketolysis**.

Synthesis of Ketone Bodies (Ketogenesis)

Synthesis of ketone bodies occurs in mitochondria, in the liver, since liver has a highly active system for the production of acetoacetate, which is the principal ketone body.

Synthesis of acetoacetate is outlined in Fig. 30.10.

- Firstly, 2 molecules of **acetyl CoA** condense and form **acetoacetyl CoA**.
- **Acetoacetyl CoA** further reacts with one molecule of **acetyl CoA** and **forms HMG CoA**.

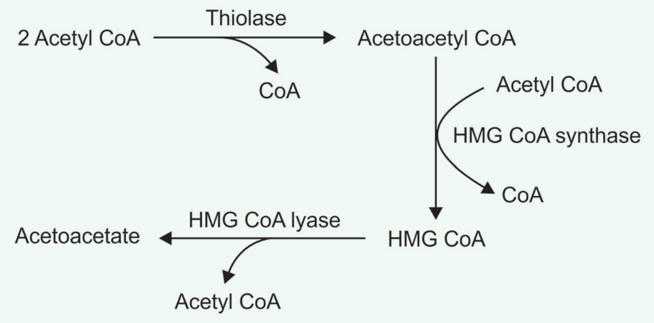


Fig. 30.10: Synthesis of acetoacetate

- Thereafter, **HMG CoA lyase** splits HMG CoA to acetyl CoA and **acetoacetate**, which is the parent ketone body.
- In the mitochondria, a part of acetoacetyl CoA **is reduced by NADH-dependent β-hydroxybutyrate dehydrogenase**, to **β-hydroxybutyrate**.
- Some of the **acetoacetate** is **decarboxylated**, spontaneously by a non-enzymatic reaction and is converted to **acetone** (Fig. 30.11).

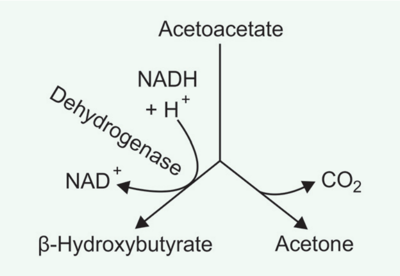


Fig. 30.11: Conversion of acetoacetate to β-hydroxybutyrate and acetone

- Both, acetoacetate and β-hydroxybutyrate are strong acids and slowly deplete alkali reserve of the body. This, in turn, results in **metabolic acidosis**, also called **ketoacidosis**.
- Under normal conditions, acetone formation is negligible but when acetoacetate accumulates, such as in **severe diabetic ketoacidosis**, amount of acetone in the blood is increased to the extent that it is detectable in breath of the patient.
- Acetoacetate and β-hydroxybutyrate, which are produced by the liver, can also be used as a source of energy in the peripheral tissues, such as kidney and muscle, during starvation.
- Although glucose is the fuel for brain in the well-nourished state but during starvation even brain starts utilizing ketone bodies.

Degradation of Ketone Bodies (Ketolysis)

- **Oxidation of β-hydroxybutyrate:** A mitochondrial enzyme **β-hydroxybutyrate dehydrogenase**, oxidizes β-hydroxybutyrate to **acetoacetate** (Fig. 30.12).

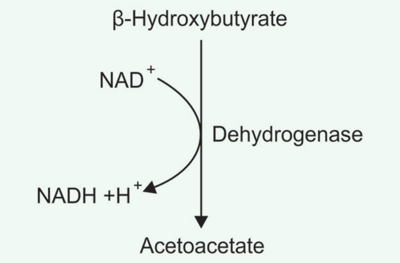


Fig. 30.12: Oxidation of β-hydroxybutyrate

- **Oxidation of acetoacetate:** Acetoacetate is oxidized after its **activation to acetoacetyl CoA**. Since liver does not contain the concerned enzyme, ketone bodies cannot be oxidized in the liver.
  - **In the first reaction**, **acetoacetate** reacts **with succinyl CoA**, in the presence of the enzyme **transacylase** (acetoacetate-succinyl CoA transferase, **also called thiophorase**), and is converted to **acetoacetyl CoA** (Fig. 30.13).

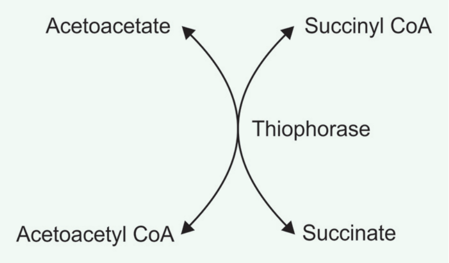


Fig. 30.13: Conversion of acetoacetate to acetoacetyl CoA

  - Thereafter, **acetoacetyl CoA is hydrolyzed to two molecules of acetyl CoA by the enzyme thiolase** (β-ketothiolase; Fig. 30.14).

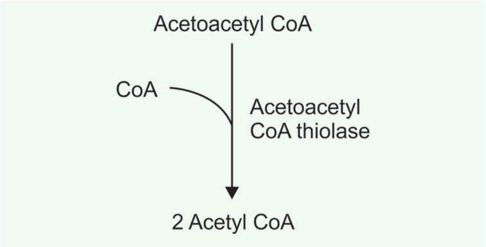


Fig. 30.14: Degradation of acetoacetyl CoA

- **Acetone** is oxidized very slowly, mainly through lungs.

LIPID PEROXIDATION AND ROLE OF ANTIOXIDANTS

In biological redox reactions, oxygen is activated by redox active metal ions, such as iron and copper, which are present in metalloenzymes and other proteins, e.g. hemoglobin, myoglobin, etc. These metal ions provide one electron at a time to oxygen and activate it to strong oxidizing form. A number of reactive oxygen species (ROS), including free radicals and non-radicals, are formed from partial reduction of molecular oxygen (Fig. 30.15).

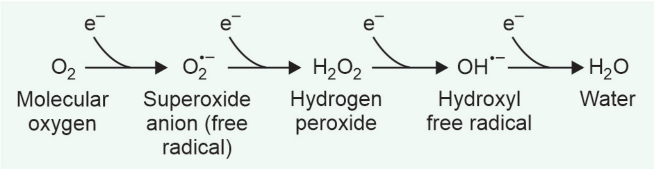


Fig. 30.15: Reduction of molecular oxygen

The first product is the superoxide anion free radical ($O_2^{\bullet}$). Reduction of superoxide yields hydrogen peroxide (H_2O_2, a non-radical ROS). Reduction of H_2O_2 causes a cleavage reaction that releases hydroxyl free radical ($OH^{\cdot}$). The end product of complete reduction of oxygen is water.

The major sources of ROS are the reaction of oxygen with de-compartmentalized metal ions, as a side reaction of electron transport or by normal enzymatic reactions of electron transport, e.g. monoamine oxidase (in dopamine metabolism), fatty acid oxidases (in the peroxisomes), etc. Basal levels of ROS are necessary for regulating processes like phagocytosis, signal transduction, gene expression and apoptosis. Any excess results in a pro-oxidative condition, known as oxidative stress.

Oxidative Stress

Various ROS are formed continuously as bye-products of aerobic metabolism through reactions with drugs and environmental toxins. When their level is increased or the level of antioxidants is diminished, it creates a condition called oxidative stress.

LIPID PEROXIDATION

Among the ROS, hydroxyl free radical ($OH^{\bullet}$) is the most reactive and damaging. Its half-life is measured in nanoseconds. It reacts with biomolecules, primarily by hydrogen abstraction and addition reactions. One of the most sensitive sites of free radical damage is the cell membrane, which is rich in readily oxidizable polyunsaturated fatty acids.

As mentioned in Chapter 3, hydroxyl free radical ($OH^{\bullet}$) extracts a hydrogen atom from a polyunsaturated fatty acid, setting off a chain of lipid peroxidation reactions. Lipid peroxides, formed in this reaction, degrade to form characteristics products such as malondialdehyde (MDA). These compounds react with proteins to form adducts and cross links, known as **advanced lipoxidation end-products** (**ALE**).

ROS also react with carbohydrates to form dicarbonyl compounds, which, in turn, react with proteins to form cross-links and adducts, known as glycoxidation products or **advanced glycation end-products** (**AGE**).

ROS also damage the genome, in the form of mutations in DNA. As a result of oxidative stress, MDA adducts, ALE and AGE, increase in several chronic conditions such as atherosclerosis, Alzheimer's disease and diabetes mellitus.

ROLE OF ANTIOXIDANTS

There are several levels of protection against oxidative damage by antioxidants, which include several enzymes and vitamins:

- **Catalase:** It is present in peroxisomes and detoxifies H_2O_2 (Fig. 30.16).

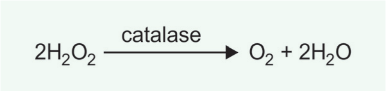


Fig. 30.16: Action of catalase

- **Superoxide dismutase (SOD):** It detoxifies $O_2^{\cdot -}$ (superoxide anion; Fig. 30.17).

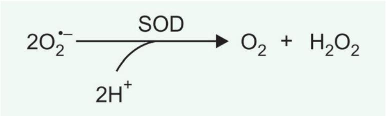


Fig. 30.17: Action of superoxide dismutase

- There are three classes of SOD – Mn.SOD found in the mitochondria, Cu-Zn.SOD widely distributed in all compartments of the cell and Fe.SOD in the extracellular matrix.
- **Glutathione peroxidase (GPx):** It reduces both H_2O_2 and lipid hydroperoxides to water and a lipid alcohol, respectively. GPx requires reduced glutathione (GSH) as a co-substrate (Fig. 30.18).

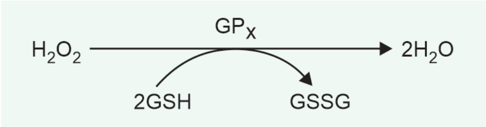


Fig. 30.18: Action of glutathione peroxidase

- **Antioxidant vitamins:** Three vitamins, i.e. vitamins A, C and E, provide the other line of defense against oxidative damage and are called antioxidant vitamins or the 'big three antioxidants'. Out of these, vitamin C (ascorbic acid) in the aqueous phase while vitamin E (α and γ-tocopherols) in the lipid phase, act as chain-breaking antioxidants.
  - Vitamin C reduces superoxide and lipid peroxyl radicals. It also has a role in reduction and recycling of vitamin E. In response to severe oxidative stress, vitamin E is maintained at constant concentration in the lipid phase until all the vitamin C is consumed.
  - Vitamin E does not propagate radical damage and is recycled.
  - Vitamin A is a lipophilic antioxidant. It is a potent singlet oxygen scavenger and protects against damage from sunlight in the retina and skin.

Consumption of food rich in vitamin C, vitamin E and β-carotene (provitamin A) has been correlated with a reduced risk for certain types of cancer and decreased frequency of certain other chronic health problems.

Important Questions

1. Outline β-oxidation of fatty acids. How much energy is produced when a molecule of palmitic acid is oxidized to acetyl CoA?
2. Outline *de novo* synthesis of fatty acid.
3. Outline cholesterol synthesis. Add a note on atherosclerosis.
4. Describe briefly:
    i. Role of carnitine in fatty acid oxidation
    ii. Catabolism of cholesterol
    iii. Synthesis of cholesterol and its regulation
5. Write notes on:
    i. Ketone bodies
    ii. Atherosclerosis
    iii. *De novo* synthesis of fatty acids
    iv. Sudden infant death syndrome
    v. Metabolic fates of acetyl CoA
    vi. Metabolic fates of cholesterol
    vii. Outline lipoprotein metabolism

Chapter

31

Metabolism of Proteins and Amino Acids

Digestion of protein releases amino acids which are absorbed by the liver. In the liver, amino acids are either used for protein synthesis or are degraded with the release of ammonia, which is used in for urea synthesis.

CATABOLISM OF AMINO ACIDS

Amino acid degradation results in removal of ammonia. There are two processes by which the **α-amino group can be removed** from amino acids. These are referred to as **deamination** and **transamination**.

DEAMINATION

Process of the **removal of α-amino group from amino acids** is referred to as deamination. Liver and kidney are the main organs which are responsible for the deamination of amino acids. Deamination may take place either by oxidative or nonoxidative process.

Oxidative Deamination

Liver and kidney contain several **enzymes which catalyze deamination** of amino acids, such as amino acid oxidases. There are two types of amino acid oxidases, called L-amino acid oxidases and D-amino acid oxidases.

L-Amino Acid Oxidases

L-amino acid oxidase is linked to FMN (the prosthetic group) and catalyzes the oxidation of L-amino acids, producing α-keto acids, ammonia and water as the final products (Fig. 31.1).

D-Amino Acid Oxidases

Some of the tissues also contain **FAD-linked D-amino acid oxidases** which **catalyze oxidation of glycine and the D-amino acids**.

Nonoxidative Deamination

Dehydratase, sulfhydrase and histidase catalyze deamination of serine, cysteine and histidine, respectively. They catalyze dehydration followed by nonoxidative deamination of the amino acid, spontaneously (Fig. 31.2).

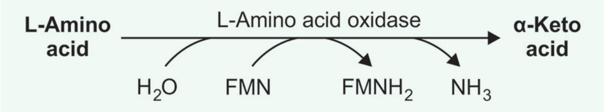

L-Amino acid
L-Amino acid oxidase
α-Keto acid
H_2O
FMN
$FMNH_2$
NH_3

Fig. 31.1: L-Amino acid oxidase reaction

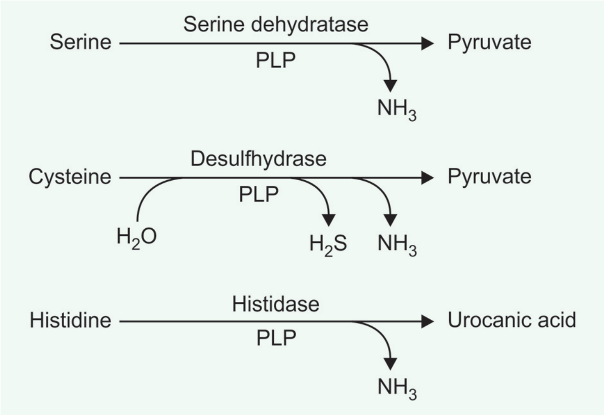

Serine
Serine dehydratase
PLP
Pyruvate
NH_3
Cysteine
Desulfhydrase
PLP
Pyruvate
H_2O
H_2S
NH_3
Histidine
Histidase
PLP
Urocanic acid
NH_3

Fig. 31.2: Nonoxidative deamination reactions

TRANSAMINATION

Transamination is a process of the **transfer of α-amino group** from **amino acid to keto acid**. As a result of it, the **amino acid is converted to keto acid** while the **keto acid is converted to amino acid** (Fig. 31.3).

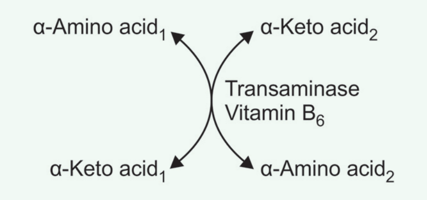

α-Amino $acid_1$
α-Keto $acid_2$
Transaminase
Vitamin B_6
α-Keto $acid_1$
α-Amino $acid_2$

Fig. 31.3: Transamination reaction

- Enzymes, which catalyze transamination, are called **transaminases** (**aminotransferases**). Although, liver contains several transaminases, most important of these are glutamate pyruvate transaminase (GPT, also called alanine **aminotransferase** or ALT) and glutamate oxaloacetate transaminase (GOT, also called **aspartate aminotransferase** or AST). Transaminases catalyzed reactions are shown in Fig. 31.4.

  An obligate amino acid or the α-keto acid which participate in these reactions, is either glutamate or α-ketoglutarate.

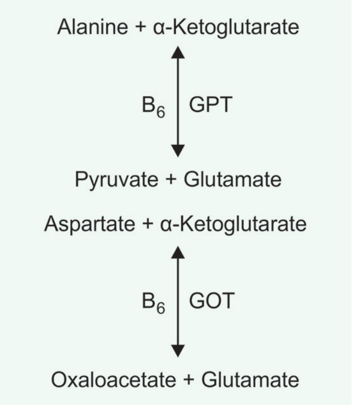


Fig. 31.4: GPT and GOT catalysed reactions

Diagnostic Significance of Transaminases

Serum GOT and GPT have diagnostic significance. They are present in large amount in cardiac muscle and liver, respectively. Although, serum levels of both the enzymes are raised in heart as well as liver diseases, **SGOT is markedly raised in myocardial infarction** while **SGPT is more increased in liver disorders** such as viral hepatitis or toxic liver necrosis (Table 31.1).

Table 31.1 Diagnostic significance of transaminases

Enzyme	Comparatively more raised in
SGOT	Cardiac disorders, e.g. myocardial infarction
SGPT	Liver disorders, e.g. viral hepatitis

DECARBOXYLATION

Decarboxylation refers to the process of the removal of carbon dioxide (CO_2) from amino acids and results in the formation of amines. It is catalyzed by the enzyme decarboxylase which requires pyridoxal phosphate as a coenzyme. For example, decarboxylation of histidine forms histamine, or tyrosine forms tyramine, etc.

TRANSAMIDATION

Transamidation (**transdeamination**) refers to the process where **transamination** is coupled with **oxidative deamination**.

As mentioned above, in the process of transamination, α-amino group (ammonia) is either transferred from glutamate (which is converted to α-ketoglutarate) or transferred to α-ketoglutarate (which is converted to glutamate). Since there are several transaminases, most of the amino acids transfer α-amino group to α-ketoglutarate and convert it to glutamate. As a result of it, glutamate pool is increased. To overcome this problem, glutamate is oxidatively deaminated by a specific enzyme, referred to as **glutamate dehydrogenase** (GDH). It is present in most of the tissues, both in cytoplasm and mitochondria. It is an anaerobic dehydrogenase and can use both NAD and NADP as a coenzyme. It catalyzes degradation as well as synthesis of glutamate. Though GDH catalyze synthesis as well as degradation of glutamate, it is important in the deamination of glutamate, which is produced as a result of transamination of most of the amino acids with α-ketoglutaric acid (Fig. 31.5).

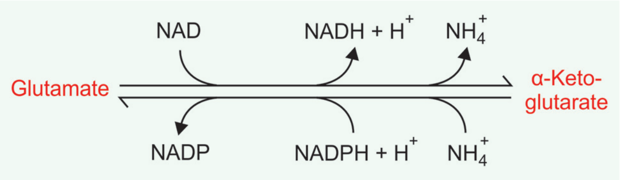


Fig. 31.5: Reactions catalyzed by glutamate dehydrogenase

METABOLIC FATES OF AMMONIA

- **In the liver**, ammonia is released from different amino acids by transamidation and is used for the synthesis of urea, which is excreted in urine.
- **In the brain**, since ammonia cannot cross blood–brain barrier, it reacts with glutamate and forms glutamine. This reaction is catalyzed by the enzyme glutamine synthetase. Glutamine is transported to the kidney where **glutaminase** removes amide group from glutamine as ammonia and forms glutamate.

Thus, ammonia from brain is transported to the kidney and is excreted as ammonium ions (NH_4^+). This is called glutamine cycle (Fig. 31.6).

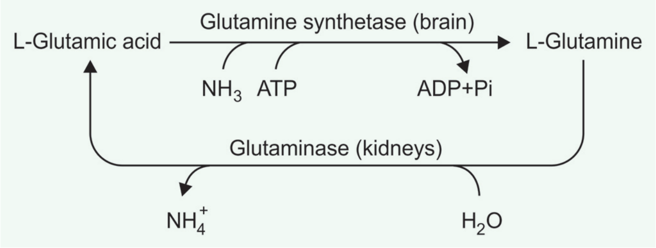


Fig. 31.6: Synthesis and degradation of glutamine

UREA CYCLE

Urea cycle is the process by which ammonia, a highly toxic water insoluble substance, is converted to urea, which is less toxic, water soluble, excretory waste product. Urea cycle occurs in the liver. Outline of urea cycle is given in Fig. 31.7.

Outline of Urea Cycle

1. In the first step, **ammonia reacts with carbon dioxide** and **forms carbamoylphosphate**, in the mitochondria. This reaction is catalyzed by the enzyme **carbamoylphosphate synthetase** (CPS), which requires N-acetylglutamate as coenzyme.
2. **Thereafter, ornithine transcarbamoylase transfers carbamoyl group from carbamoylphosphate to ornithine** and forms **citrulline**.
3. In the next step, in the presence of the enzyme **argininosuccinate synthetase**, **citrulline** combines with L-aspartate and **forms argininosuccinate**.
4. Subsequently, **argininosuccinase** hydrolyzes **argininosuccinate** to **arginine and fumarate**. Fumarate, which is released in this reaction, enters mitochondria where it is converted to oxaloacetate via citric acid cycle. Hence, urea cycle is coupled with the Krebs cycle, both of which were discovered by Sir Hans Krebs and his coworkers. Thus, urea cycle is also called urea bicycle (Krebs-Henseleit cycle).
5. In the last reaction, **arginine** is hydrolyzed **by the enzyme arginase**, to **ornithine** and **urea**. Ornithine is transported to mitochondria and re-enters the cycle.

Urea cycle is the mechanism for the excretion of nitrogen in humans and other primates. In the synthesis of urea, two atoms of nitrogen are derived from two different sources, i.e. from NH_3 and the amino group of aspartate.

Inborn Errors of Metabolism of Urea

A defect in any enzyme of the urea cycle may result in an inborn error. In general, since ammonia is very toxic, a metabolic disorder of urea cycle may result in coma and may be potentially fatal (Table 31.2).

Table 31.2 Inborn errors of metabolism of urea cycle

Enzyme	Disorder
Carbamoylphosphate synthetase	Hyperammonemia type I
Ornithine transcarbamoylase	Hyperammonemia type II
Argininosuccinate synthetase	Citrullinemia
Argininosuccinase	Argininosuccinic aciduria
Arginase	Hyperargininemia

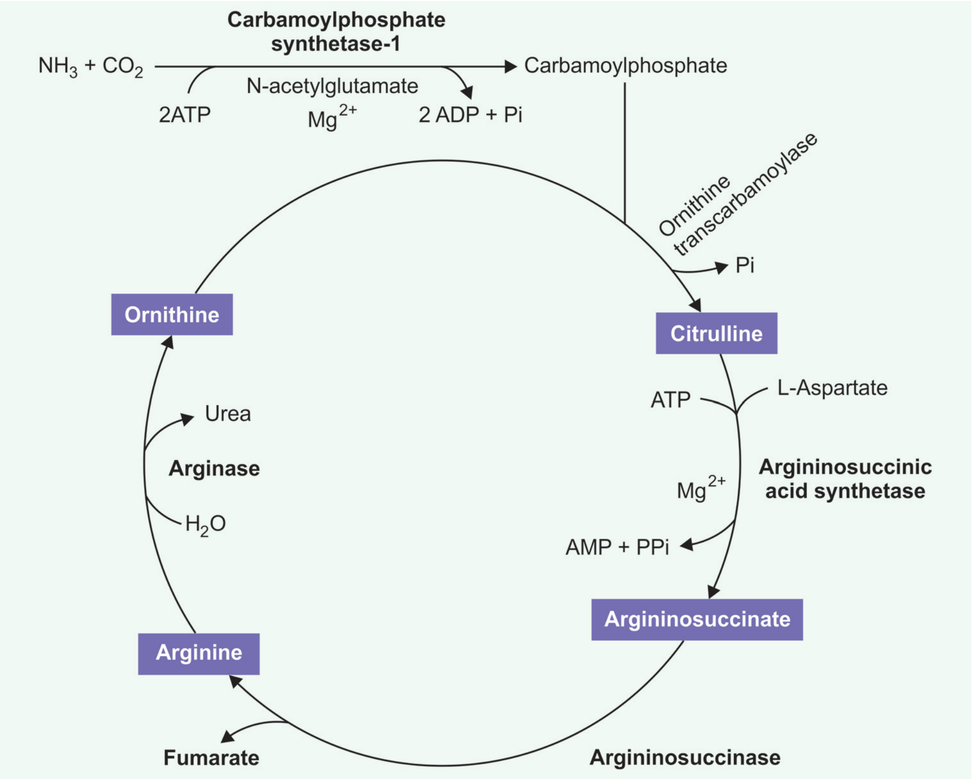

Carbamoylphosphate synthetase-1
NH3 + CO2
Carbamoylphosphate
N-acetylglutamate
2ATP
Mg2+
2 ADP + Pi
Ornithine transcarbamoylase
Pi
Ornithine
Citrulline
ATP
L-Aspartate
Urea
Arginase
Argininosuccinic acid synthetase
Mg2+
H2O
AMP + PPi
Argininosuccinate
Arginine
Fumarate
Argininosuccinase

Fig. 31.7: Urea cycle

METABOLISM OF SOME AMINO ACIDS

METABOLISM OF GLYCINE

Glycine is the simplest amino acid found in proteins. It is a nonessential amino acid and is glucogenic in nature.

Outline of Metabolism of Glycine

- Glycine is degraded to CO_2 and **ammonia** by the enzyme **glycine cleavage complex** (Fig. 31.8).

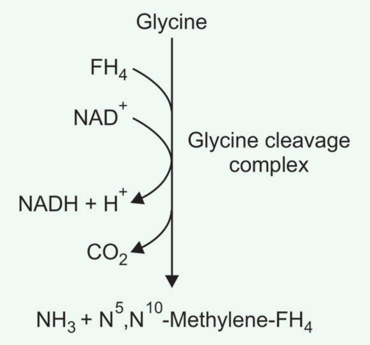

Glycine
FH_4
NAD^+
Glycine cleavage complex
$NADH + H^+$
CO_2
$NH_3 + N^5,N^{10}$-Methylene-FH_4

Fig. 31.8: Catabolism of glycine to CO_2 and NH_3

- Some of the glycine is also degraded by oxidative deamination, by the enzyme **glycine oxidase** and is converted to **glyoxylate**.

  Glyoxylate can either be transaminated back to glycine or is oxidized to oxalate (Fig. 31.9).

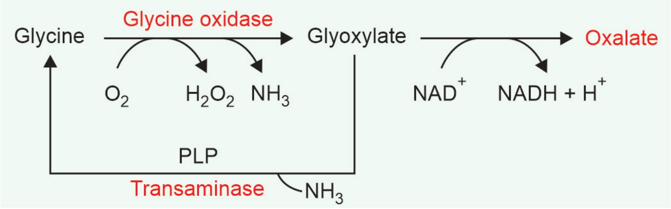

Glycine
Glycine oxidase
Glyoxylate
Oxalate
O_2
H_2O_2
NH_3
NAD^+
$NADH + H^+$
PLP
Transaminase
NH_3

Fig. 31.9: Conversion of glycine to oxalate

Specialized Products Formed From Glycine

Several specialized products are formed from glycine (Fig. 31.10).

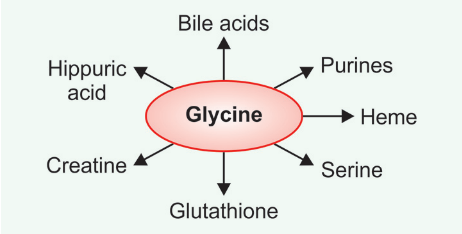

Bile acids
Hippuric acid
Purines
Glycine
Heme
Creatine
Serine
Glutathione

Fig. 31.10: Specialized products formed from glycine

1. **Serine:** Glycine is converted to serine. Formation of serine from glycine is a reversible reaction. It is catalyzed by the enzyme **serine hydroxymethyl-transferase**, which requires pyridoxal-5-phosphate and N^5,N^{10}-methylenetetrahydrofolate (N^5,N^{10}-methylene•FH_4; Fig. 31.11).

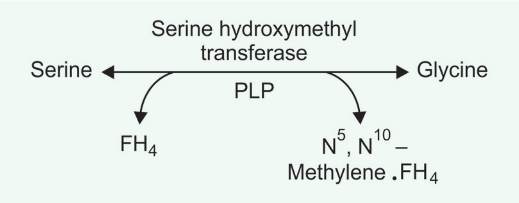

Serine hydroxymethyl transferase
Serine
Glycine
PLP
FH_4
N^5, N^{10} – Methylene .FH_4

Fig. 31.11: Conversion of glycine to serine

The demand for glycine or serine and the availability of N^5,N^{10}-methylene•FH_4 determine direction of the reaction.

2. **Purine ring:** Glycine is an important contributor in the biosynthesis of a purine ring. It contributes nitrogen and both the carbons, which form N_7, C_4 and C_5 of a purine ring (Fig. 31.12).

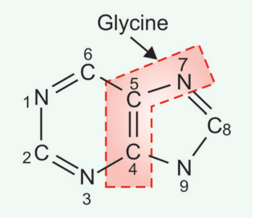

Glycine
1 N
2 C
N 3
C 4
C 5
C 6
N 7
C_8
N 9

Fig. 31.12: Contribution of glycine in the synthesis of a purine ring

3. **Heme:** Glycine joins with succinyl CoA and forms δ-aminolevulinic acid, a precursor of heme. This reaction is catalyzed by aminolevulinic acid (ALA) synthase (Fig. 31.13).

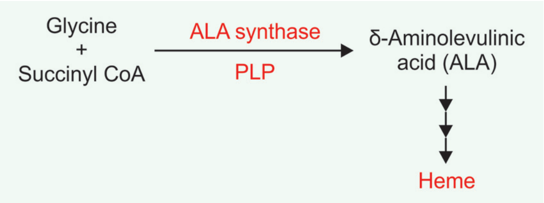

Glycine + Succinyl CoA
ALA synthase
PLP
δ-Aminolevulinic acid (ALA)
Heme

Fig. 31.13: Contribution of glycine in the synthesis of heme

4. **Creatine:** Glycine also contributes in the biosynthesis of creatine, a reservoir of energy in muscle (Fig. 31.14).

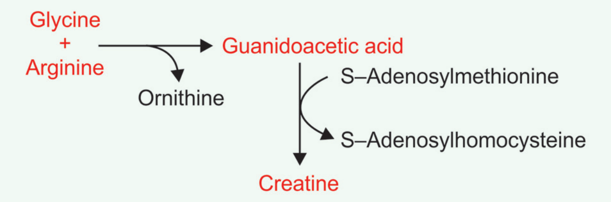


Fig. 31.14: Contribution of glycine in the synthesis of creatine

5. **Glutathione:** Glycine is also a constituent of the tripeptide glutathione.
6. **Bile acid:** Glycine conjugates with cholic acid and forms glycocholic acid (a bile acid).
7. **Hippuric acid:** Glycine is also used in the detoxication of benzoic acid, which is excreted in urine as hippuric acid.

Inborn Errors of Glycine Metabolism

Hyperglycinemia

Deficiency of glycine cleavage complex results in a condition, referred to as nonketotic hyperglycinemia. It is characterized by severe mental retardation. Neurological complications of the disease are due to hyperglycinemia, since glycine is an inhibitory neurotransmitter. Many patients do not survive infancy.

Primary Hyperoxaluria

Excessive production of oxalate, due to transaminase deficiency (Fig. 31.9), results in a condition referred to as primary hyperoxaluria. Oxalate forms calcium oxalate crystals, high urinary excretion of which may lead to renal calculi (kidney stones).

METABOLISM OF PHENYLALANINE AND TYROSINE

As men, and most animals, cannot synthesize an aromatic ring, hence, phenylalanine is dietary essential. Under normal conditions, dietary phenylalanine that is not incorporated into proteins is converted to tyrosine.

Outline of Metabolism of Phenylalanine and Synthesis of Tyrosine

Phenylalanine is **converted to tyrosine** in the liver. This reaction is catalyzed by the enzyme phenylalanine hydroxylase (Fig. 31.15).

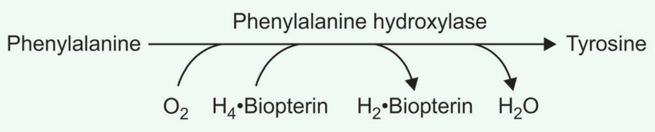


Fig. 31.15: Conversion of phenylalanine to tyrosine

Inborn Error of Phenylalanine Metabolism

Inborn error of phenylalanine metabolism is referred to as **phenylketonuria** (PKU). It is an autosomal recessive disorder due to the deficiency of hepatic **phenylalanine hydroxylase**. As a result of it, phenylalanine cannot be converted to tyrosine, which, in turn, becomes dietary essential.

Due to deficiency of phenylalanine hydroxylase, phenylalanine is degraded by an alternate pathway:

- Phenylalanine is first converted to phenylpyruvic acid by the enzyme phenylalanine transaminase.
- Subsequently, phenylpyruvate is also reduced to phenyllactic acid as well as decarboxylated to phenylacetic acid (Fig. 31.16).

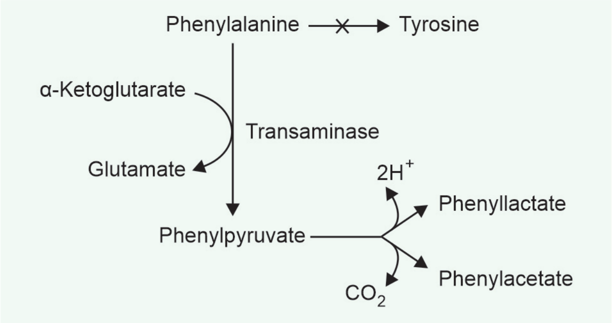


Fig. 31.16: Conversion of phenylalanine to phenylketoacids in phenylketonuria

Due to increased urinary excretion of phenylpyruvic acid (a phenylketone), this disease is called phenylketonuria. Increased levels of phenylketoacids in blood cause mental retardation and convulsions. Such infants, patients with PKU, are given diets low in phenylalanine. Under this condition, tyrosine becomes dietary essential.

Tyrosine, since, can be synthesized from phenylalanine, it is not dietary essential.

Outline of Metabolism of Tyrosine

- In the first step, tyrosine is converted to **p-hydroxyphenylpyruvate** by **tyrosine aminotransferase** (tyrosine transaminase). It is an inducible enzyme whose synthesis is increased by glucocorticoids as well as tyrosine.

- In the next step, a copper-containing enzyme, **p-hydroxyphenylpyruvate oxidase**, converts **p-hydroxyphenylpyruvate** to **homogentisic acid**.
- p-Hydroxyphenylpyruvate oxidase is a complex, which requires ascorbic acid and catalyses decarboxylation, oxidation, migration of the side chain as well as its hydroxylation.
- Subsequently, an iron-containing enzyme, **homogentisic acid oxidase, oxidizes homogentisic acid**.
- Subsequent reactions are catalyzed by **maleylacetoacetate isomerase** and **fumarylacetoacetate hydrolase**. This, in turn, results in the formation of the end product of tyrosine catabolism, i.e. **fumarate** and **acetoacetate** (Fig. 31.17).

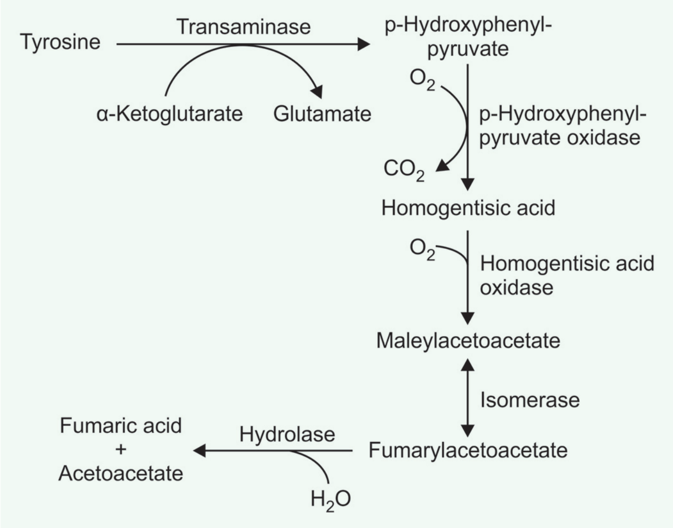

Tyrosine
Transaminase
p-Hydroxyphenyl-pyruvate
α-Ketoglutarate
Glutamate
O_2
p-Hydroxyphenyl-pyruvate oxidase
CO_2
Homogentisic acid
O_2
Homogentisic acid oxidase
Maleylacetoacetate
Isomerase
Fumaric acid + Acetoacetate
Hydrolase
Fumarylacetoacetate
H_2O

Fig. 31.17: Degradation of tyrosine in the liver

Specialized Products Formed from Phenylalanine and Tyrosine

As discussed above, phenylalanine is converted to tyrosine, which serves as a precursor of a number of specialized products, in different tissues (Fig. 31.18).

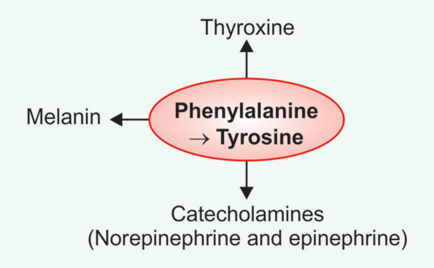

Thyroxine
Melanin
Phenylalanine → Tyrosine
Catecholamines
(Norepinephrine and epinephrine)

Fig. 31.18: Specialized products formed from tyrosine

Synthesis of Catecholamines

In adrenal medulla, tyrosine is converted to norepinephrine (noradrenaline) and epinephrine (adrenaline), the two hyperglycemic hormones, secreted by the gland.

- First, tyrosine is converted to dihydroxyphenylalanine (**DOPA**) by the enzyme **tyrosine hydroxylase**.
- Thereafter, **DOPA-decarboxylase converts DOPA to dopamine**, which enters the storage granules as an active neurotransmitter.
- In the next step, **dopamine** is converted to **norepinephrine by dopamine-β-hydroxylase**.
- **Norepinephrine** is a hormone, present in sympathetic nerve endings. Its synthesis is regulated by plasma tyrosine in the brain.
- In the next step, methylation of **norepinephrine** by the enzyme **phenylethanolamine-N-methyltransferase** (PNMT), converts it to **epinephrine**. In this reaction active methionine (S-adenosylmethionine) is used as a methyl donor (Fig. 31.19).

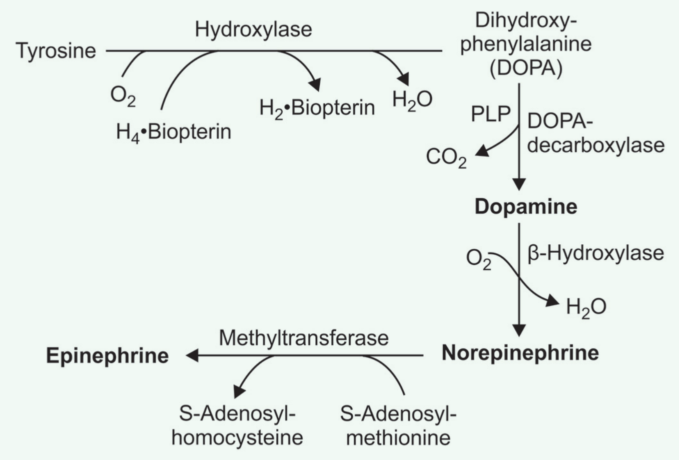

Tyrosine
Hydroxylase
Dihydroxy-phenylalanine (DOPA)
O_2
H_4•Biopterin
H_2•Biopterin
H_2O
PLP
DOPA-decarboxylase
CO_2
Dopamine
O_2
β-Hydroxylase
H_2O
Epinephrine
Methyltransferase
Norepinephrine
S-Adenosyl-homocysteine
S-Adenosyl-methionine

Fig. 31.19: Synthesis of catecholamines from tyrosine in the adrenal medulla

Catecholamines

Aromatic amines with a catechol ring (3,4-dihydroxyphenyl ring), i.e. dopamine, norepinephrine and epinephrine, are collectively called catecholamines.

They have general modulatory effects on overall brain functions, such as mood and arousal.

Dopamine

It is an intermediate in the synthesis of norepinephrine. It is a neurotransmitter in nerves, that interconnect nuclei of the basal ganglia in brain, and controls voluntary movement. Damage to these nerves causes Parkinson's disease, which is characterized by tremor and difficulties in initiating and controlling movement.

Norepinephrine

It is a major transmitter in sympathetic nervous system. Stimulation of these nerves is responsible for various features of **fight or flight** response such as stimulation of heart rate, sweating and vasoconstriction in skin and bronchodilation.

Epinephrine

It is produced by adrenal medulla under the influence of acetylcholine-containing nerves analogous to the sympathetic pre-ganglionic nerves. It has stimulatory effect on glycogen metabolism in the liver.

Both, norepinephrine and epinephrine are released from the storage vesicles in adrenal medulla, in response to stress, exercise, cold and low levels of blood glucose. They increase degradation of glycogen and triacylglycerols as well as increase blood pressure and output of the heart.

Synthesis of Thyroid Hormones

In the thyroid gland, iodination of tyrosyl residues of thyroglobulin results in the synthesis of thyroid hormones (Fig. 31.20).

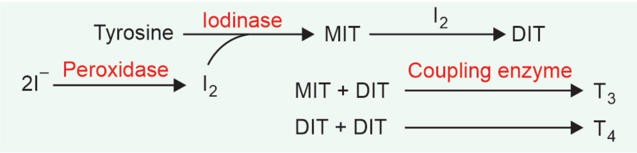


Fig. 31.20: Conversion of the tyrosyl residues in thyroglobulin to thyroid hormones in the thyroid gland

- In the first step of **iodination**, iodine is first activated by peroxidase.
- Subsequently, iodinase catalyzes iodination of the tyrosyl residues of thyroglobulin and converts these tyrosine molecules to monoiodotyrosine (MIT) and diiodotyrosine (DIT).
- Thereafter, **coupling** of either a molecule of MIT with DIT or two molecules of DIT, forms triiodothyronine (T_3) and thyroxine (tetraiodothyronine or T_4), respectively. This is an intramolecular process and hormones are synthesized within the protein chain of the thyroglobulin molecule.

Synthesis of Melanin

In melanocytes, tyrosine is converted to melanin.

- In the first step, **tyrosine is converted to DOPA** and **then to dopaquinone** by the enzyme **tyrosinase** (a copper-containing protein).
- Subsequently, dopaquinone **forms melanin**, a brown-black pigment, found in skin, hair and retina (Fig. 31.21).

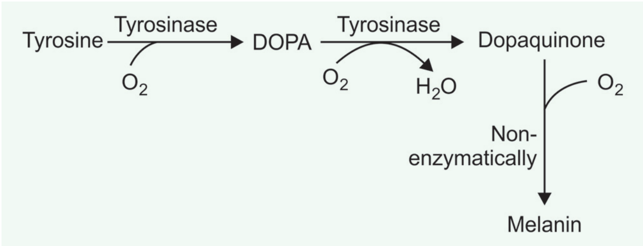


Fig. 31.21: Synthesis of melanin from tyrosine in melanocytes

Inborn Errors of Tyrosine Metabolism

Tyrosinemias

A complete, or partial, deficiency of **tyrosine aminotransferase** results in **tyrosinemia**, which, in turn, leads to the accumulation and excretion of tyrosine and its metabolites. There are two types of tyrosinemias:

Tyrosinemia type I

It is also called **hepatorenal tyrosinemia**. It is caused by the deficiency of **fumarylacetoacetate hydrolase** and is characterized by liver failure, renal tubular dysfunction, rickets and polyneuropathy.

Tyrosinemia type II

It is also called **oculocutaneous tyrosinemia**. It is due to the deficiency of **tyrosine transaminase**. It results in eye and skin lesions, and mental retardation.

Alkaptonuria

Alkaptonuria is due to hereditary deficiency of the enzyme **homogentisic acid oxidase**. The individuals excrete homogentisic acid in their urine. Homogentisic acid, in turn, gets auto-oxidized and is, subsequently, polymerized to form intensely dark substance, referred to as **alkapton**.

Dark urine is the only consequence of this condition, in early life. Homogentisate is slowly oxidized and deposited in the bone, connective tissue and other organs. This condition is called **ochronosis** because of ochre color of the pigment.

Albinism

Lack of tyrosinase in the melanocytes affects the synthesis of melanin and leads to the formation of white patches on the skin and iris in the eyes. This condition is referred to as **albinism**.

Lack of the pigment in the skin makes the albinos more sensitive to sunlight and increases the risk of carcinoma of skin. Lack of the pigment in the eyes contributes to photophobia.

Various disorders of phenylalanine/tyrosine metabolism are shown in Table 31.3.

Table 31.3 Disorders of phenylalanine/tyrosine metabolism

Metabolic disorder	Deficient enzyme
Phenylketonuria	Phenylalanine hydroxylase
Tyrosinemia type I	Fumarylacetoacetate hydrolase
Tyrosinemia type II	Tyrosine transaminase
Alkaptonuria	Homogentisic acid oxidase
Albinism	Tyrosinase

METABOLISM OF TRYPTOPHAN

Tryptophan is an essential amino acid containing indole ring.

Outline of Metabolism of Tryptophan

Main pathway for the degradation of tryptophan is called kynurenine-anthranilate pathway. It is outlined in Fig. 31.22.

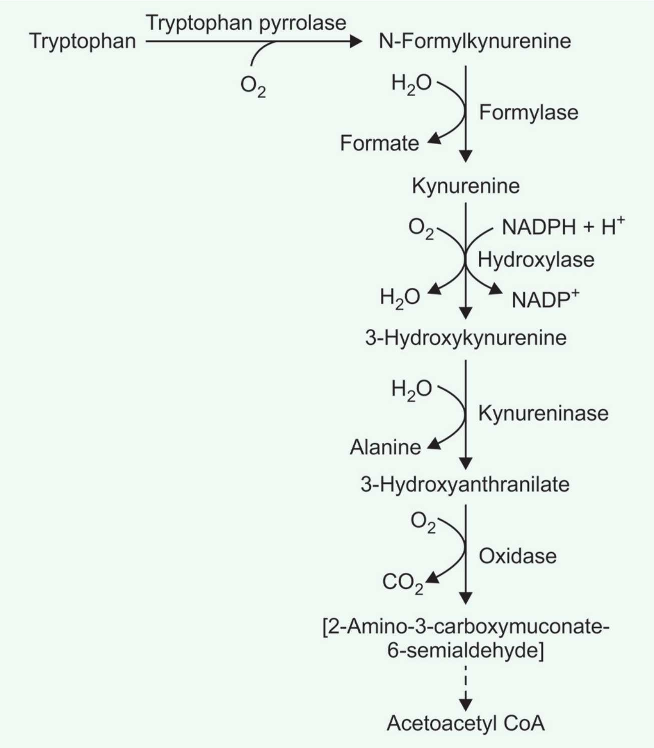

Tryptophan
Tryptophan pyrrolase
O_2
N-Formylkynurenine
H_2O
Formylase
Formate
Kynurenine
O_2
NADPH + H^+
Hydroxylase
H_2O
$NADP^+$
3-Hydroxykynurenine
H_2O
Kynureninase
Alanine
3-Hydroxyanthranilate
O_2
Oxidase
CO_2
[2-Amino-3-carboxymuconate-6-semialdehyde]
Acetoacetyl CoA

Fig. 31.22: Degradation of tryptophan via kynurenine-anthranilate pathway in the liver

- It is operative in the liver, where degradation of **tryptophan** starts with the **opening of the indole ring**, leading to the formation of **N-formylkynurenine**. The reaction is catalyzed by a heme-containing enzyme, **tryptophan dioxygenase**, also called **tryptophan pyrrolase**. The enzyme is found in the liver and is induced by glucocorticoids as well as glucagon.
- In the next step, **formylkynurenine** is converted to **kynurenine** with the removal of formate, by the enzyme **formamidase** (formylase).
- Thereafter, kynurenine is **hydroxylated** to produce **3-hydroxykynurenine** by **kynurenine hydroxylase**. This enzyme is inhibited by estrogen and, thus, women are more susceptible to pellagra (niacin deficiency disease).
- In the next step, with the cleavage of **alanine** from the side chain by **kynureninase**, **3-hydroxykynurenine** is converted to **3-hydroxyanthranilate**. This enzyme requires pyridoxal-5-phosphate (vitamin B_6) as a coenzyme.
- Subsequent oxidation of **3-hydroxyanthranilate** by **3-hydroxyanthranilate oxidase** forms an unstable intermediate **2-amino-3-carboxymuconate-6-semialdehyde**.
- Through a number of steps, finally it is converted to glutarate and eventually to **acetoacetyl CoA**.

Specialized Products Formed from Tryptophan

Various specialized products formed from tryptophan are shown in Fig. 31.23.

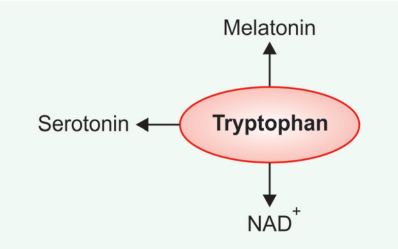

Melatonin
Serotonin
Tryptophan
NAD^+

Fig. 31.23: Specialized products formed from tryptophan

Synthesis of NAD

- Firstly, in the liver and some other tissues, nonenzymatic cyclization, of **aminocarboxymuconic semialdehyde** (formed in the kynurenine-anthranilate pathway), converts it to **quinolinic acid**.
- Thereafter, **quinolate phosphoribosyltransferase** (QPRT) transfers a ribonucleotide moiety from phosphoribosylpyrophosphate (PRPP). Subsequent decarboxylation of which leads to the synthesis of

nicotinate mononucleotide (a derivative of niacin) and finally to NAD (Fig. 31.24).

Sixty mg of tryptophan is converted to 1 mg of niacin. In B_6 deficiency quinolinic acid is not converted to niacin but to nicotinamide and its N^1-methyl derivative (N^1-methylnicotinamide). Vitamin B_6 deficiency, thus, results in urinary excretion of large quantities of these metabolites.

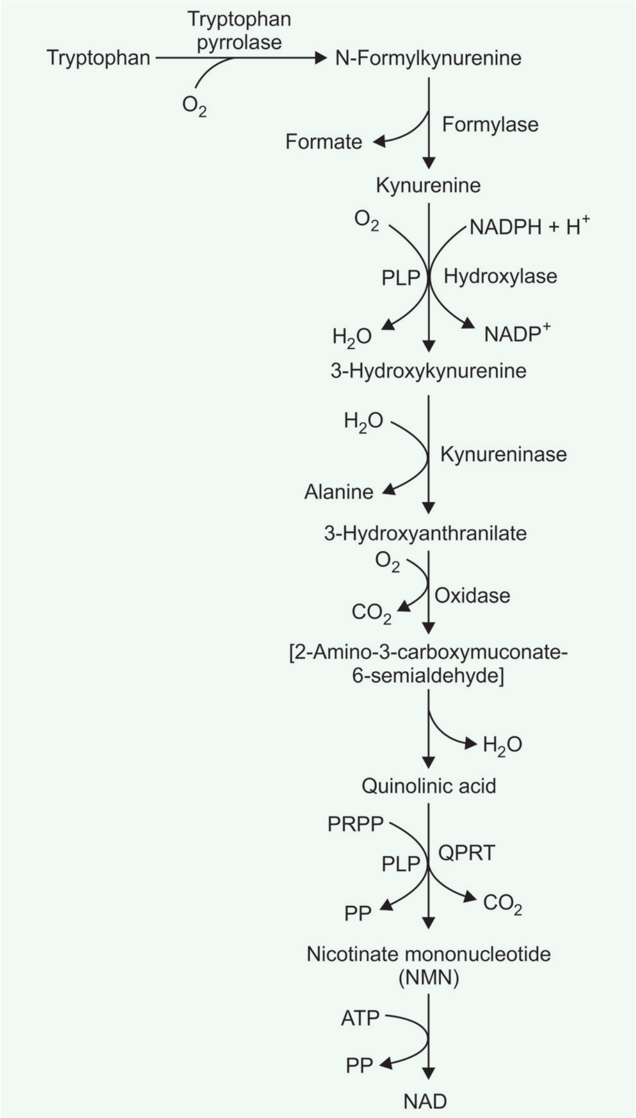

Tryptophan pyrrolase
Tryptophan
O_2
N-Formylkynurenine
Formylase
Formate
Kynurenine
O_2
NADPH + H^+
PLP
Hydroxylase
H_2O
$NADP^+$
3-Hydroxykynurenine
H_2O
Kynureninase
Alanine
3-Hydroxyanthranilate
O_2
Oxidase
CO_2
[2-Amino-3-carboxymuconate-6-semialdehyde]
H_2O
Quinolinic acid
PRPP
PLP
QPRT
PP
CO_2
Nicotinate mononucleotide (NMN)
ATP
PP
NAD

Fig. 31.24: Synthesis of NAD from tryptophan

Synthesis of Serotonin

- In platelets, brain and intestinal epithelium, tryptophan is hydroxylated by **tryptophan hydroxylase**, to form **5-hydroxytryptophan**.
- 5-Hydroxytryptophan is subsequently decarboxylated to **5-hydroxytryptamine**, also called **serotonin**, by a **decarboxylase**, which also requires B_6 for its activity (Fig. 31.25).

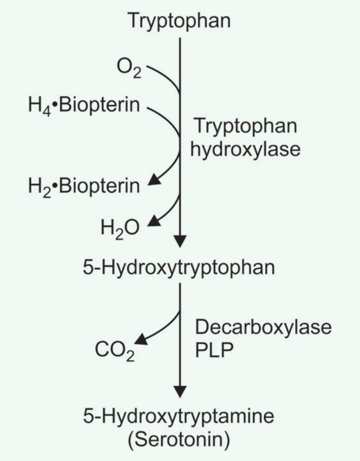

Tryptophan
O_2
H_4•Biopterin
Tryptophan hydroxylase
H_2•Biopterin
H_2O
5-Hydroxytryptophan
Decarboxylase PLP
CO_2
5-Hydroxytryptamine (Serotonin)

Fig. 31.25: Synthesis of serotonin from tryptophan

Serotonin is a neurotransmitter (in the brain), a vasoconstrictor and bronchoconstrictor. Serotonin-producing tumor cells in the intestine result in a condition referred to as **malignant carcinoid syndrome**, also called **argentaffinoma**, because this tumor arises from the intestinal argentaffin cells, that normally secrete serotonin. There is an increased production of serotonin that causes flushing, anxiety and gastrointestinal upset.

Serotonin is normally catabolized to **5-hydroxyindoleacetic acid** (**5-HIAA**). In this condition, urinary excretion of 5-HIAA is increased by more than 50 fold and its estimation is used to establish the diagnosis.

Synthesis of Melatonin

An **acetyltransferase**, present in the pineal gland and retina, converts **serotonin** into **N-acetyl-5-methoxytryptamine**, also called **melatonin** (Fig. 31.26).

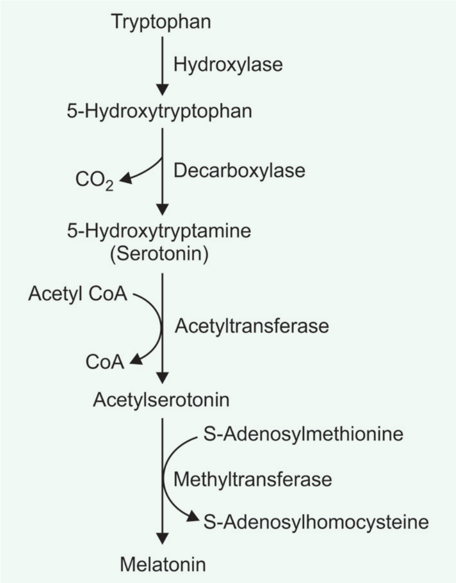

Tryptophan
Hydroxylase
5-Hydroxytryptophan
CO_2
Decarboxylase
5-Hydroxytryptamine (Serotonin)
Acetyl CoA
Acetyltransferase
CoA
Acetylserotonin
S-Adenosylmethionine
Methyltransferase
S-Adenosylhomocysteine
Melatonin

Fig. 31.26: Synthesis of melatonin from tryptophan

Melatonin is a sleep-inducing substance and maintains circadian rhythm of sleep wake cycle. It also inhibits synthesis and secretion of other neurotransmitters, such as dopamine and GABA.

METABOLISM OF METHIONINE

Methionine is an essential amino acid and cannot be formed in the body. It is a glycogenic amino acid, as the end product of its catabolism is succinyl CoA. Methionine also acts as a lipotropic factor.

Outline of Metabolism of Methionine

Metabolism of methionine is outlined in Fig. 31.27.

- The most important step in the metabolism of methionine is catalyzed by **adenosyltransferase**, which converts methionine to **S-adenosylmethionine**, also called active methionine.
- **Active methionine** acts as a **methyl carrier** and **donor**, and helps in the **synthesis of several methylated products**. Methylation reactions are catalyzed by specific methyltransferases, which transfer a methyl group from SAM to an acceptor molecule. This reaction is important in the formation of methylated products of biological significance (Table 31.4).

Table 31.4 Use of S-adenosylmethionine (SAM) as a methyl donor

Methyl acceptor	Product formed	Methylated products of biological significance
Ethanolamine	Choline	Choline-containing phospholipids
Guanidoacetate	Creatine	Creatine phosphate (a high-energy reservoir)
N-acetylserotonin	Melatonin	Neurotransmitter
Norepinephrine	Epinephrine	Catecholamine
Epinephrine	Metanephrine	Metabolite of epinephrine
Bases in DNA and RNA, and amino acids in proteins	Methylated bases in nucleic acids, and methylated amino acids in proteins	Regulation of gene expression

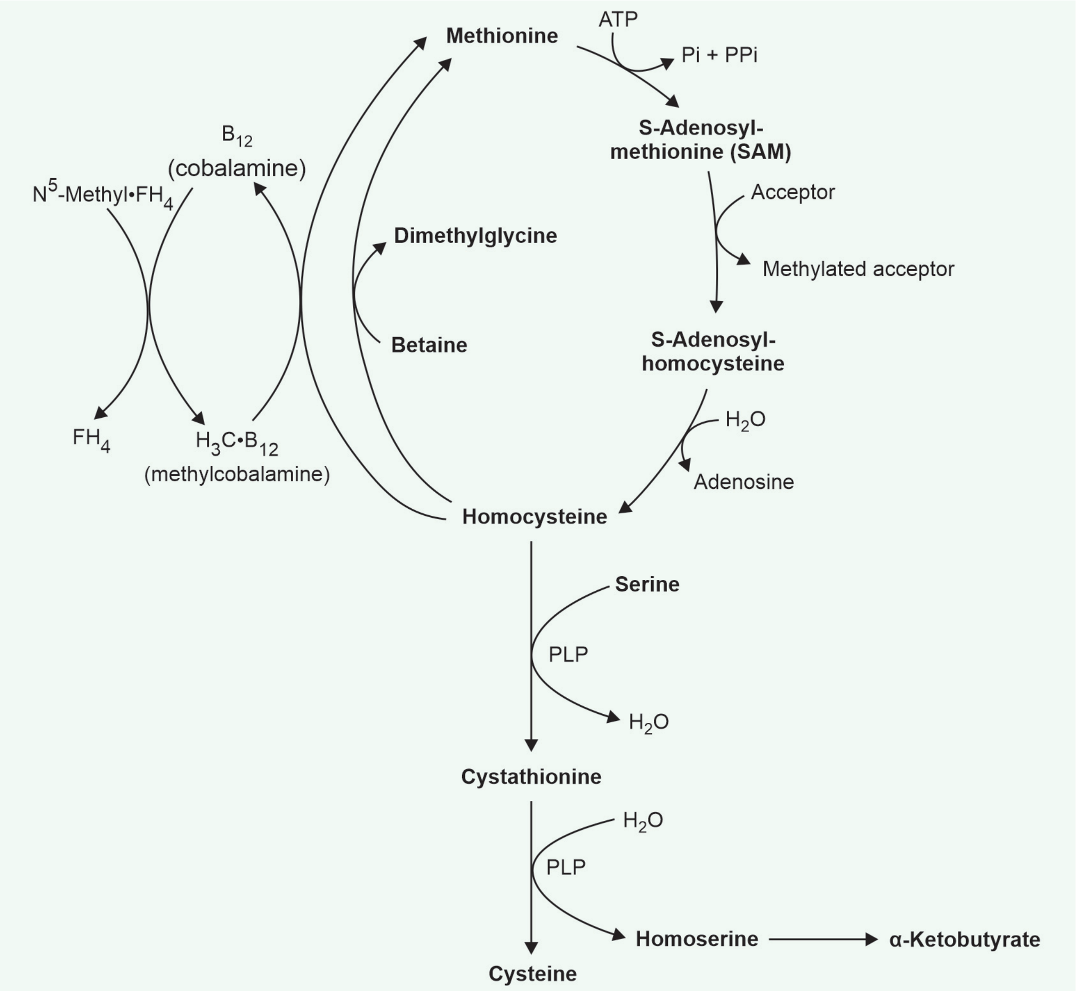

Methionine
ATP
Pi + PPi
S-Adenosyl-methionine (SAM)
Acceptor
Methylated acceptor
S-Adenosyl-homocysteine
H_2O
Adenosine
Homocysteine
Serine
PLP
H_2O
Cystathionine
H_2O
PLP
Homoserine
α-Ketobutyrate
Cysteine
Dimethylglycine
Betaine
B_{12}
(cobalamine)
N^5-Methyl•FH_4
FH_4
$H_3C•B_{12}$
(methylcobalamine)

Fig. 31.27: Conversion of methionine to S-adenosylmethionine (active methionine), homocysteine and cysteine

- S-Adenosylmethionine is also used in the synthesis of polyamines, i.e. spermidine and spermine.
- After the transfer of the methyl group, S-adenosylmethionine is converted to **S-adenosylhomocysteine**.
- In the next step, S-adenosylhomocysteine is converted to **homocysteine**.
- When cells need to resynthesize methionine, **homocysteine methyltransferase** transfers a methyl group from **N^5-methyltetrahydrofolate** (N^5-methyl•FH_4) and convert homocysteine to methionine.

Specialized Products Formed from Methionine

Methionine is a precursor of **cysteine**, which is synthesized as follows:

- After removal of the active methyl group, by a methyltransferase (as discussed above), **S-adenosylmethionine** is converted to **S-adenosylhomocysteine**.
- Thereafter, **adenosylhomocysteinase**, cleaves the adenosyl moiety (a molecule of adenosine) and forms **homocysteine**, which has one carbon more than cysteine.
- In the next step, **homocysteine** is coupled with **serine** and forms **cystathionine**. The reaction is catalyzed by the enzyme **cystathionine synthase**, which also requires pyridoxal phosphate as a coenzyme.
- In the next step, **cystathionine** is cleaved by a splitting enzyme, **cystathione lyase**, also called **cystathionase**, and forms **cysteine** and **α-ketobutyrate**.

Inborn Errors of Methionine Metabolism

Hypermethioninemia

Methionine adenosyltransferase deficiency results in a condition called **hypermethioninemia**. Blood methionine levels are increased.

Homocystinurias

It includes a group of autosomal recessive conditions which result in accumulation of homocysteine in blood, called **hyperhomocysteinemia**, and its excretion in urine, referred to as **homocysteinuria**. It may be a result of:

Cystathionine-β-synthase Deficiency

It causes increased plasma methionine and homocysteine levels and their increased excretion in urine. As a result of it plasma cysteine level is reduced. It is associated with mental retardation, skeletal deformities involving the spine (vertebra) and thorax, seizures and dislocation of the lens. There also occurs a defect in the synthesis of collagens, since homocysteine may react with, and block, lysyl groups of collagens.

Cystathioninuria

It is due to lack of the enzyme β-cystathioninase and results in mental retardation.

Acquired Hyperhomocystinemia

It is due to nutritional deficiency of B-complex vitamins, such as methyl cobalamine, tetrahydrofolate, pyridoxal phosphate, which are required as coenzymes in many reactions in methionine metabolism. This condition is associated with increased generation of reactive oxygen species, causing endothelial cell damage, an initial event in atherosclerosis. Further, homocysteine can also form homocysteine thiolactone (a highly reactive intermediate), which thiolates free amino groups in LDL and cause their aggregation. These lipid aggregates are endocytosed by macrophages and form atheromas.

METABOLISM OF CYSTEINE

Cysteine, as discussed above, can be synthesized from methionine, hence, cysteine is a nonessential amino acid.

Outline of Metabolism of Cysteine

Transamination

By transamination cysteine is changed to pyruvic acid.

- Cysteine is transaminated and converted to β-mercaptopyruvic acid.
- In the next step, it is changed to pyruvic acid.
- Sulfur is removed either as H_2S or as sulfite.

Decarboxylation

On decarboxylation, cysteine is converted to β-mercaptoethanolamine, which is used in the synthesis of coenzyme A.

Specialized Products Formed from Cysteine

Synthesis of Taurine

- **Cysteine** is oxidized by the enzyme **cysteine dioxygenase** to form **cysteine sulfinate**.
- The latter is decarboxylated to **hypotaurine** (2-aminoethane sulfate), which is oxidized to form **taurine**.

Synthesis of Active Sulfate

- **Taurine**, as synthesized above, undergoes transamination with α-ketoglutaric acid and is converted to **bisulfite** and pyruvate.

- In the next step, **sulfite oxidase** oxidizes **bisulfate**, which is converted to **sulfate**.
- In the next step, **sulfate** in the presence of ATP and the enzyme **adenosinephosphosulfate pyrophosphorylase**, is converted to **3′-phosphoadenosine-5′-phosphosulfate** (PAPS), also called **active sulfate**.

Active sulfate is used as a source of sulfate for the formation of sulfate esters of steroids, alcohols and phenols.

Synthesis of SH-containing Peptides

Cysteine is also used in the synthesis of other sulfur-containing compounds of physiological importance such as insulin, CoA, glutathione and vasopressin.

METABOLISM OF CYSTINE

Cystine or dicysteine can be converted to two molecules of **cysteine** by **cystine reductase**, which requires NADH$^+$ (Fig. 31.28).

Cysteine is a glycogenic amino acid. It is required for the synthesis of several proteins, particularly, the synthesis of scleroproteins, found in the hair and keratin of nails, etc. Cysteine also helps in maintaining the structure of protein molecules by forming –S–S– bridges between the peptide chains, such as in insulin.

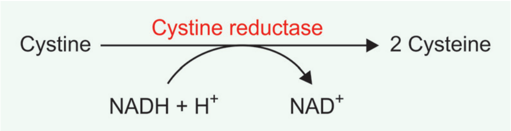


Fig. 31.28: Conversion of cystine to cysteine

Inborn Errors of Cystine Metabolism

Cystinosis

It is an autosomal recessive hereditary disorder, characterized by defective, carrier-mediated transport of cystine and is also called **cystine storage disease**. There is an excessive deposition of cystine crystals in the lysosomes in various tissues including kidney, bone marrow, cornea, conjunctiva and peripheral leukocytes. There is generalized aminoaciduria, glycosuria and, in some cases, rickets or osteomalacia. The condition may appear, both in children as well as adults. Cystine crystals may result in loss of kidney function, usually causing renal failure, within ten years.

METABOLISM OF HISTIDINE

Histidine is a semiessential amino acid. It is glycogenic in nature, since it is converted to glutamate and finally, through α-ketoglutarate, to glucose. It is also important in one-carbon metabolism.

Outline of Metabolism of Histidine

Histidine degradation occurs mainly in the liver (Fig. 31.29).

- In the first step, **ammonia** is removed and histidine is converted to **urocanic acid** (urocanate). This reaction is catalyzed by the enzyme **histidase** (histidine ammonia lyase).
- In the next step, there is internal oxidation of the imidazole ring and reduction of the side chain with the addition of water. This reaction is catalyzed by the enzyme **urocanase** (urocanic acid hydratase) and requires pyridoxal-5-phosphate as a coenzyme.
- In the next step, imidazole ring is hydrolytically cleaved between nitrogen-3 and carbon-4 by the enzyme **imidazolonepropionase** (a hydrolase), forming **formiminoglutamate** (**FIGLU**).
- In the presence of **tetrahydrofolic acid** (FH_4), **glutamate formiminotransferase** removes the formimino group as N^5-formiminotetrahydrofolic acid, and glutamate is formed (Fig. 31.29).

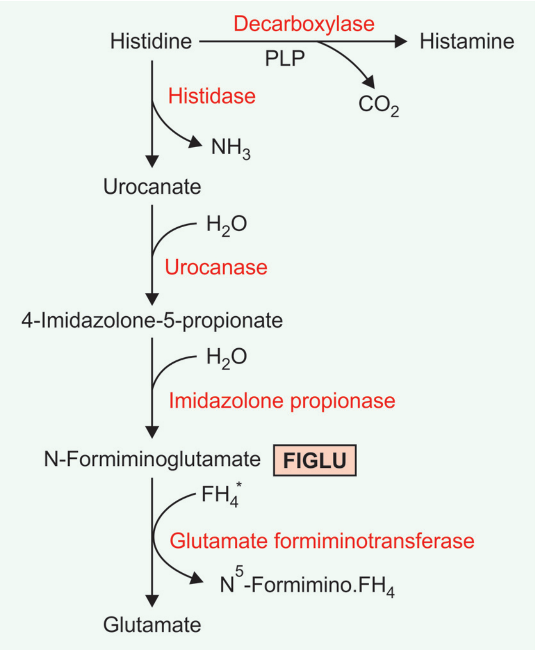


Fig. 31.29: Outline of metabolism of histidine

FIGLU Excretion Test

In patients with folate deficiency, conversion of FIGLU to glutamate is impaired, as a result of which FIGLU is excreted in urine. This is a diagnostic test of folate deficiency, after a test dose of histidine.

Specialized Products Formed from Histidine

Histamine

Histamine is a pharmacologically active compound. It is a vasodilator and reduces blood pressure. It is also a stimulant of gastric juice secretion. Allergic reactions increase liberation of histamine.

Inborn Errors of Histidine Metabolism

Histidinemia

This is a hereditary disorder of histidine metabolism, due to deficiency of the enzyme histidase. It is characterized by elevated levels of histidine in plasma and urine. Some of the histidine may be diverted to the alternative pathway, as a result of which imidazolepyruvate, imidazolelactate and imidazole-acetate are excreted in urine. Besides speech defects during childhood, there is retardation of mental development.

METABOLISM OF BRANCHED CHAIN AMINO ACIDS

Valine, leucine and isoleucine are the three essential amino acids, which have aliphatic, nonpolar side chain and are referred to as branched chain amino acids (BCAA). These are essential amino acids and have different metabolic fates.

Outline of the Metabolism of Branched Chain Amino Acids

Metabolism of the branched chain amino acids (BCAA, i.e. valine, leucine and isoleucine) though produces different products, the first three steps in their degradation are similar:

- The first step in the oxidation of BCAA is the **removal of the α-amino group**, by transamination, forming the corresponding α-keto acids. This reaction is catalyzed by the enzyme BCAA-**aminotransferase**.
- In the next step, dehydrogenation of the branched chain α-keto acids is carried out by **branched chain α-keto acid dehydrogenase complex**, which, in turn, results in the loss of a carboxylic group and forms CoA thioesters of BCAA.
- The enzyme, branched chain α-keto acid dehydrogenase complex has 3 subunits, called **α-keto acid decarboxylase**, **transacylase** and **dihydrolipoyl-dehydrogenase**. Further, it requires NAD$^+$, FAD, TPP, lipoic acid and coenzyme A.
- In the next step, branched chain-acyl CoA thioesters are oxidized to the corresponding **α,β-unsaturated branched chain-acyl CoA thioesters**.
- These thioesters, thereafter, follow unique pathways to the amphibolic end products, which determine that **valine is glucogenic**, **leucine is ketogenic** and **isoleucine is**, both, **ketogenic and glucogenic** (Fig. 31.30).

Inborn Errors of Branch Chain Amino Acids Metabolism

Maple Syrup Urine Disease

Inborn error of branch chain amino acids metabolism is referred to as **maple syrup urine disease** (MSUD). It is caused by a deficiency of **branched chain α-keto acid**

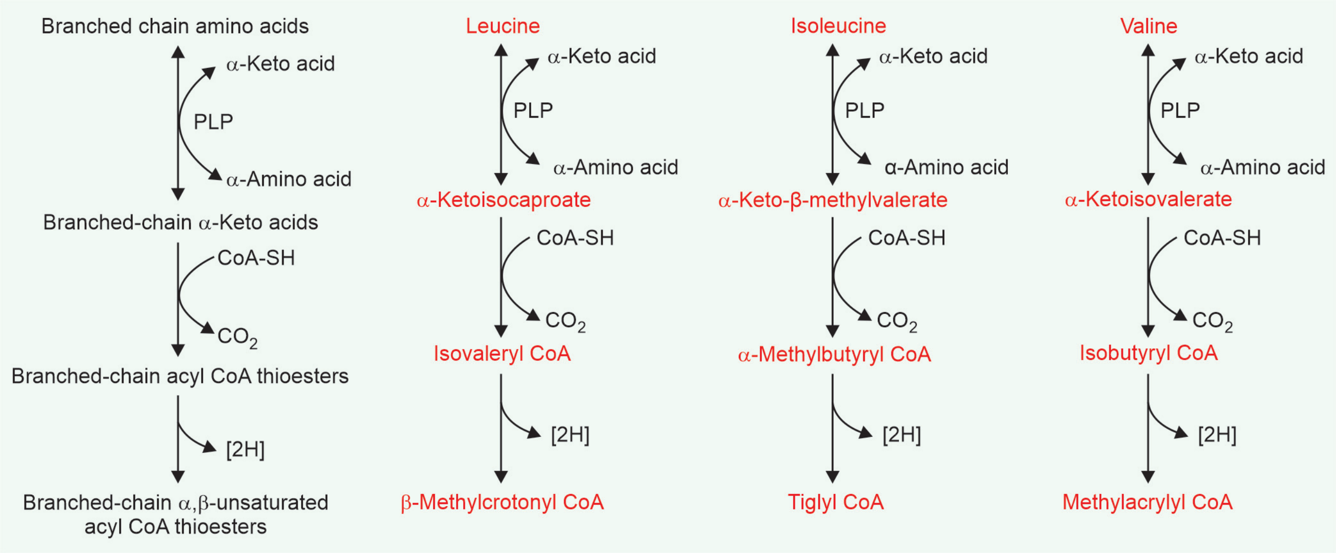

Branched chain amino acids
α-Keto acid
PLP
α-Amino acid
Branched-chain α-Keto acids
CoA-SH
CO2
Branched-chain acyl CoA thioesters
[2H]
Branched-chain α,β-unsaturated acyl CoA thioesters
Leucine
α-Keto acid
PLP
α-Amino acid
α-Ketoisocaproate
CoA-SH
CO2
Isovaleryl CoA
[2H]
β-Methylcrotonyl CoA
Isoleucine
α-Keto acid
PLP
α-Amino acid
α-Keto-β-methylvalerate
CoA-SH
CO2
α-Methylbutyryl CoA
[2H]
Tiglyl CoA
Valine
α-Keto acid
PLP
α-Amino acid
α-Ketoisovalerate
CoA-SH
CO2
Isobutyryl CoA
[2H]
Methylacrylyl CoA

Fig. 31.30: Outline of metabolism of branched chain amino acids

dehydrogenase complex (BCKDC), leading to a build-up of branched chain amino acids (leucine, isoleucine and valine) and their keto acids in blood and urine. The enzyme complex consists of four subunits and the disease can result from mutation in any of the genes that code for the enzyme subunits.

- This disease is named for the **presence of** sweet-smelling urine, with an odor similar to that of **maple syrup**. The smell is sometimes stronger in the ear wax of an affected individual.
- Infants with this disease seem healthy at birth but, if left untreated, suffer severe brain damage and, eventually, die because **high levels of the keto acids of the BCAA** are toxic to the brain and other organs.
- From early infancy, symptoms of the condition include poor feeding, vomiting, dehydration, lethargy, hypotonia, seizures, hypoglycemia, ketoacidosis, pancreatitis, coma and neurological decline.
- Keeping MSUD under control requires careful monitoring of blood chemistry and involves, both a special diet and frequent testing.
- A diet with minimal levels of the amino acids valine, leucine and isoleucine must be maintained in order to prevent neurological damage.
- Some patients may also improve with the administration of the high doses of thiamine, a cofactor of the enzyme that causes the condition.

CREATINE METABOLISM

Creatine is present in the muscle, liver, kidney and brain. It is stored in muscle as creatinephosphate (phosphocreatine).

Synthesis of Creatine

Three amino acids, i.e. **glycine**, **arginine** and **methionine**, are used for the **synthesis of creatine** (Fig. 31.31).

- First, **guanidino group of arginine** is transferred to **glycine** and forms **guanidoacetate**. This reaction is catalyzed by the enzyme **arginine-glycine transaminidase**, in the kidney.
- Subsequently, **guanidoacetate methyltransferase** transfers **methyl group from S-adenosylmethionine**, in the liver and forms creatine.
- Creatine is stored in muscle as **phosphocreatine**.

Conversion of Creatine to Creatine Phosphate and Its Significance

Creatine phosphate is the form in which high energy phosphate is stored in the muscle. It is synthesized from creatine by the transfer of a high energy phosphate group from ATP. This reaction is catalyzed by creatinephosphokinase (CPK), also called creatine kinase (CK).

- **During resting condition** creatine phosphate is utilized to phosphorylate ADP and creatine is released as creatinine.
- **During exercise** ATP is used as a source of energy and is converted to ADP.

Synthesis of Creatinine and Its Significance

Creatinine is the **anhydride** form of creatine. As mentioned above, it is formed in the muscle from creatine phosphate by a nonenzymatic reaction, irreversibly.

- Creatinine is one of the major **nonprotein nitrogenous** excretory products. Nearly 1.0–1.5 g of creatinine is excreted in the urine, in 24 hours.
- Its total excretion is independent of the amount of dietary protein but is related to **muscle mass**.
- Serum creatinine as well as amount of creatinine excreted by an individual remains constant from day to day and by calculating creatinine clearance, one can **determine renal function, in terms of glomerular filtration rate**.
- Only traces of creatine are normally present in the urine. Large quantities may be excreted in muscular dystrophies, during starvation and pregnancy.

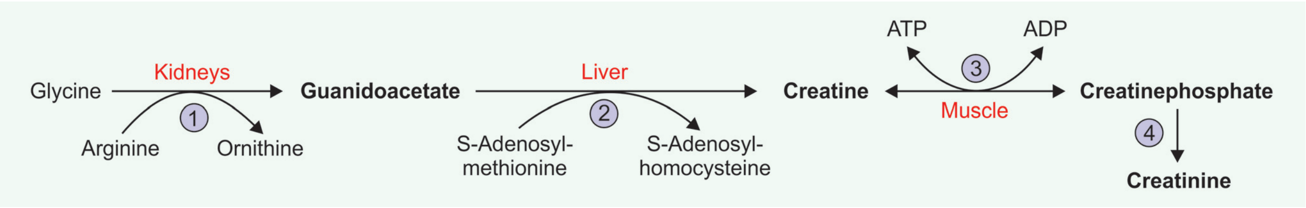


Fig. 31.31: Synthesis of creatine and creatinine: (1) Transaminidase; (2) Methyltransferase; (3) Creatine phosphokinase; (4) Nonenzymatic reaction

Important Questions

1. Outline urea cycle. What is the site of urea synthesis? What is the need for urea synthesis in the body?
2. Describe various mechanisms of the removal of ammonia from amino acids.
3. Explain how tyrosine is metabolized. Describe specialized products formed from tyrosine.
4. Discuss briefly:
    i. Reactions of urea cycle and its inborn errors
    ii. Transamination and its importance
    iii. Metabolism of phenylalanine
5. Write notes on:
    i. Alkaptonuria
    ii. Phenylketonuria
    iii. Deamination

Chapter

32

Nucleic Acids

Nucleic acids are the macromolecules, which occur in cells in association with basic proteins, such as histones and protamines. They are strongly acidic in nature, and are negatively charged at physiological pH.

Two types of nucleic acids are found in nature, these are called **deoxyribonucleic acid** (**DNA**) and **ribonucleic acid** (**RNA**). These are essential for the storage and transfer of genetic information as well as protein synthesis.

DEOXYRIBONUCLEIC ACID

Deoxyribonucleic acid (**DNA**) stores genetic information in the cell and is called the **chemical basis of heredity**. DNA is a large molecule, which occurs in various sizes, with few hundred to several million nucleotides. It is a double-stranded polymer of deoxyribonucleotides, which are linked together by phosphodiester bonds.

STRUCTURE OF DNA

Watson and Crick described various features of DNA, hence the most commonly referred structure of DNA is called **Watson-Crick model** of DNA (Fig. 32.1).

- DNA is a polynucleotide of **deoxyadenylate** (**dAMP**), **deoxyguanylate** (**dGMP**), **deoxycytidylate** (**dCMP**) and **thymidylate** (**TMP**).
- Information content of DNA resides in the **sequence**, in which various deoxyribonucleotides are arranged.
- The polymer possesses a **polarity**. Its left-hand terminal has a free hydroxyl group or phosphate and is called 5′-terminus. On the other hand, the right-handed terminal is referred to as 3′-terminus.
- DNA is a **double-stranded** molecule, i.e. it has two strands (polynucleotide sequences or chains), which run **antiparallel** to each other. Whereas, its one strand runs in the 5′→3′ direction while the other one runs in the opposite direction, i.e. in 3′→5′ direction.
- Both the polynucleotide chains are wound around a common axis, forming **right-handed helix**, which is nearly 20Å in diameter.
- This helix has a **pitch of 34 Å** with 10 base-pairs (bp) per turn.
- The two strands of the helix are held together by **hydrogen bonds**, which are formed between a purine and a pyrimidine. This base pairing is very specific, as A is always linked to T with two hydrogen bonds while G is linked to C with three hydrogen bonds.
- Thus, a DNA molecule has equal number of adenine and thymine residues as well as guanine and

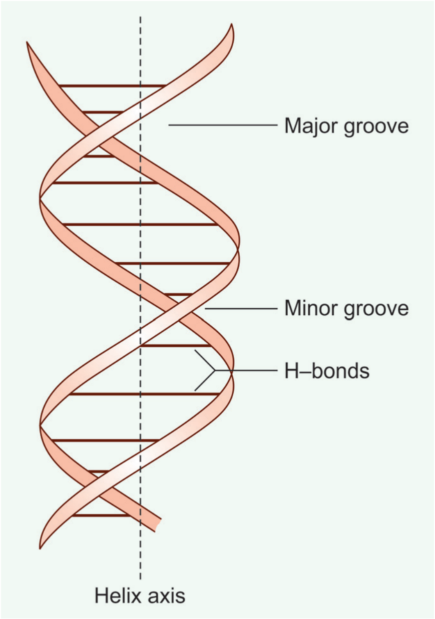


Fig. 32.1: Watson-Crick model of DNA

cytosine residues. This relationship is known as **Chargaff's rule**.

- Bases occupy core of the helix while sugar-phosphate chain runs along the periphery. This, in turn, forms two grooves of unequal width. They are called the **major groove** and the **minor groove**.

TYPES OF DNA

There are 3 different structural forms of DNA, which are referred to as B-DNA, A-DNA and Z-DNA.

B-DNA

This is the most common, biologically active form of DNA that has structural features described by Watson and Crick, i.e. it has two antiparallel polynucleotide strands, which have a right-handed helix.

Bases occupy core of the helix. Each of its base pair has nearly same width that accounts for symmetry of the molecule. Helix has 10 base pairs (bp) per turn and a pitch of 34Å.

When dehydrated, B-DNA undergoes reversible conformational changes, i.e. it flattens and becomes wide. It is referred to as A-DNA.

A-DNA

A-DNA has 11 bp per turn and a pitch of 28 Å, with an axial hole. Further, plane of its base pair is tilted by 20°, with respect to its axis. It has a deep major groove and a very shallow minor groove.

Z-DNA

Z-DNA has 12 bp per turn, a pitch of 45 Å and a deep major groove but no identifiable minor groove. It has a sequence of complementary polynucleotides with alternating purines and pyrimidines, such as poly d(GC).d(AT) or poly d(AC).d(GT) chains, with a Z (zig zag) conformation.

RIBONUCLEIC ACIDS

Three main classes of RNA are known to occur in cells. They are known as **messenger RNA**, **ribosomal RNA** and **transfer RNA**. Though all the three types of RNA are involved in protein biosynthesis, they differ from each other with respect to their size, composition, location within the cell and functions.

TYPES OF RNA

Messenger RNA

Messenger RNA (mRNA) is a group of RNA, which is most heterogeneous. They are highly elongated but short-lived molecules, which are synthesized within the nucleus, as a result of transcription.

The mRNA transcript has $5' \rightarrow 3'$ polarity, which is complementary to the coding strand of gene (DNA molecule) with the only difference that mRNA contains uracil in place of thymine, which is found in DNA (Fig. 32.2).

The mRNA conveys message (information) from gene to the protein-synthesizing machinery. This information is present in mRNA in the form of sequences of nucleotides, as triplets, called codons. Each mRNA serves as a template on which a specific sequence of amino acids is polymerized to form a specific protein, the ultimate product of gene. mRNAs are, generally, stable in eukaryotes but quite unstable in prokaryotes.

In mammalian cells, the transcription product of DNA is a large molecule called **heterogeneous nuclear RNA (hnRNA)** that undergoes post-transcriptional changes and gets converted to mRNA, which is released into cytosol and is used for protein biosynthesis.

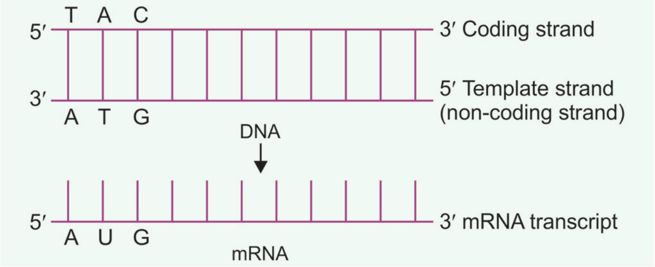


Fig. 32.2: Representation of DNA and the corresponding mRNA

Transfer RNA

Transfer RNA (tRNA) molecules are much smaller in size and consist of 75 to 90 ribonucleotides only. Total tRNA comprises about 15 per cent of the cellular RNA. tRNA is also known as soluble RNA (sRNA) because it is found in the soluble fraction of the cell.

There are about 50 different types of tRNAs, each one of which fulfils its role as an adapter molecule and has a sequence of three nucleotides, called anticodon. Each tRNA binds an amino acid and locates itself at the correct codon, for the translation of information. This information is present in the sequence of nucleotides on mRNA and is translated into the sequence of amino acids, thereby, forming a peptide chain.

Various tRNAs, though differ from each other with respect to their nucleotide sequences, as a class have many common features:

- The nucleotide sequence (primary structure) allows extensive folding and intramolecular base-pairing to form a secondary structure, which gives it a cloverleaf-like structure.
- All tRNA molecules, in addition to the four bases, i.e. A, G, C and U, also contain several unusual (modified) bases, e.g. 7-methylguanine, thymine, dihydrouracil, pseudouracil, hypoxanthine, etc.
- All tRNA molecules have different intramolecular foldings, called arms:
  - **Amino acid acceptor arm:** It has 7 base-paired stem that has CCA sequence in the 5′→3′ direction at the terminal. Its adenosyl moiety (A) accepts the particular amino acid. In this process, the carboxyl group of amino acid forms an ester linkage with the 3′-hydroxyl group of the adenosyl moiety.
  - **Anticodon arm:** It has 5 base-pairs and a loop. Its loop has a triplet, i.e. a sequence of three nucleotides called anticodon. Hence, this arm is called anticodon arm. The anticodon has a sequence which is complementary to the sequence of three nucleotides of the codon. It is responsible for the specificity of the **tRNA** molecule.
  - **DHU arm:** It has an unusual base called dihydrouracil (DHU or UH2), in its loop. Hence, it is called DHU arm. Besides DHU, it has 3 to 4 basepairs.
  - **TψC arm:** It has a sequence of thymine (T), pseudouracil (ψ) and cytosine (C) in its loop. Hence, it is called TψC arm. Besides, it also has 5 basepairs.
  - In addition to the above, each tRNA also has one small arm, called **extra arm**, which has a small loop of 3–5 basepairs (Fig. 32.3).

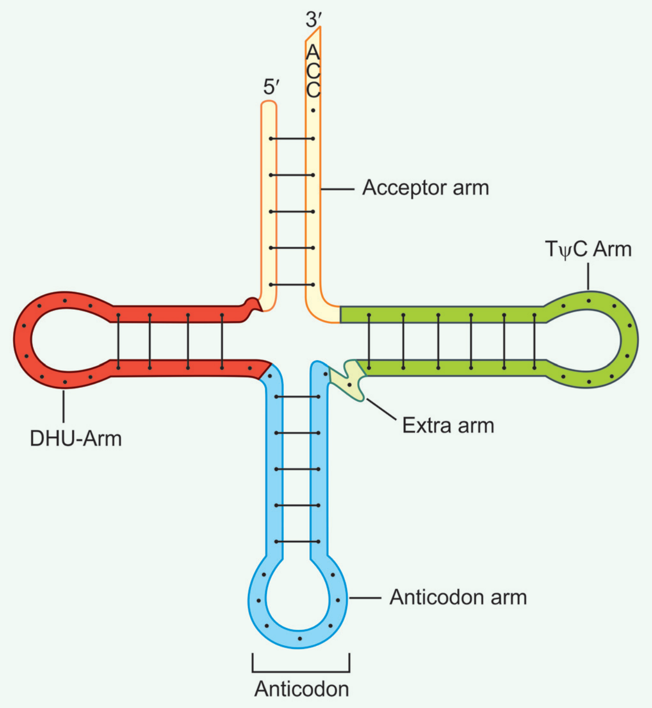


Fig. 32.3: tRNA

Ribosomal RNA

Ribosomal RNA (rRNA) molecules are present in ribosomes, in association with several polypeptides.

Ribosomes are found in cytosol of the cell and contain nearly 80% of the total RNA, found in the cell. Both, mRNA and tRNA interact on ribosomes, to translate the information, which is transcribed from gene, into a specific protein.

Mammalian ribosomes are very complex in nature with a sedimentation velocity of 80S. Each ribosome has 2 subunits which are referred to as **large subunit** (60S) and **small subunit** (40S; Fig. 32.4).

The large subunit contains 3 different types of rRNA, which are designated as 5S, 5.8S and 28S rRNA. It also has more than 50 polypeptides. The small subunit has 18S rRNA and nearly 30 polypeptides.

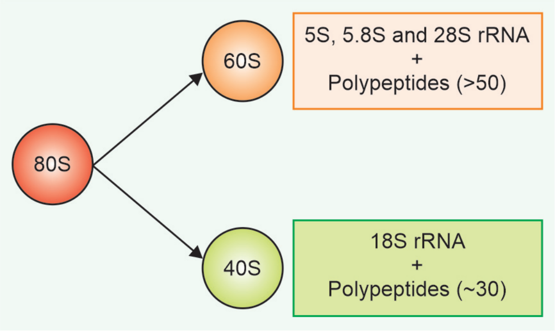


Fig. 32.4: Mammalian ribosome particles and their contents

DIFFERENCES BETWEEN DNA AND RNA

DNA differs from RNA in several aspects (Table 32.1).

Table 32.1 Differences between DNA and RNA

	DNA	RNA
Sugars	Deoxyribose	Ribose
Bases	A, G, C and T	A, G, C and U
Strands	Double-stranded	Usually single-stranded
Purine/ pyrimidine ratio	A = T and G = C Also, A + G = C + T	No ratio

BIOLOGICAL SIGNIFICANCE OF NUCLEIC ACIDS

Nucleic acids perform various functions, such as that:

- DNA makes copies of it and thus, transfers genetic information from one generation to the next. This process is known as **replication**.
- DNA also determines properties of a living cell and regulates biological information by controlling protein synthesis through RNA. This information is passed from DNA to RNA, by the process referred to as **transcription**. Thereafter, the sequence of bases from **RNA** is translated into the sequence of amino acids in a protein. This process is referred to as **translation**.

Thus, **DNA serves as a template**, both, **for replication** of information into the daughter DNA as well as **for transcription** of the information into RNA and its subsequent translation into protein (Fig. 32.5).

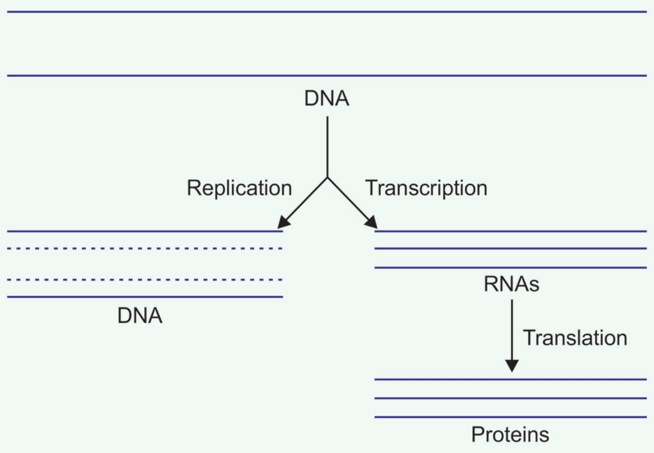


Fig. 32.5: Functions of DNA: Replication and transcription, and of RNAs: Translation

NUCLEOTIDES

Repeated monomer units, called **nucleotides**, are the **building blocks** of nucleic acids, as amino acids to proteins or monosaccharides to polysaccharides. A **nucleotide** has 3 constituents, i.e. **a nitrogenous base**, **a pentose sugar** and **phosphoric acid**.

NITROGENOUS BASES FOUND IN NUCLEIC ACIDS

Nitrogenous bases, found in nucleotides, are the derivatives of either a **purine** or a **pyrimidine**.

Purines

A purine is a **nine-member ring**, containing **5 carbons** and **4 nitrogen atoms** (Fig. 32.6).

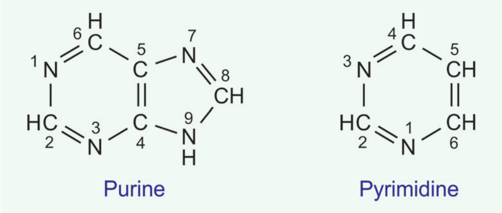


Fig. 32.6: Purine and pyrimidine rings

There are two **purine** bases found in nucleic acids. These are referred to as **adenine** (6-aminopurine) and **guanine** (2-amino-6-oxypurine).

Pyrimidines

A pyrimidine is a **six-member ring**, having **4 carbons** and **2 nitrogen atoms**. Pyrimidine bases found in nucleic acids include **cytosine** (2-oxy-4-aminopyrimidine), **uracil** (2,4-dioxypyrimidine) and **thymine** (2,4-dioxy-5-methylpyrimidine, also referred as 5-methyluracil (Fig. 32.7).

Out of the three pyrimidines, only two are found in a nucleic acid. Usually, **cytosine** and **uracil** are found in **RNA**, while **cytosine** and **thymine** are found in **DNA**.

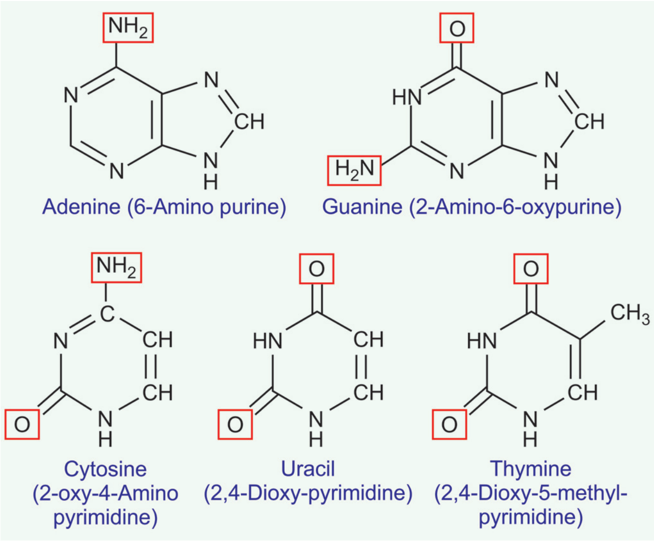

Adenine (6-Amino purine)
Guanine (2-Amino-6-oxypurine)
Cytosine (2-oxy-4-Amino pyrimidine)
Uracil (2,4-Dioxy-pyrimidine)
Thymine (2,4-Dioxy-5-methyl-pyrimidine)

Fig. 32.7: Purine and pyrimidine bases found in nucleic acids

PENTOSE SUGARS FOUND IN NUCLEIC ACIDS

Presence of the pentose sugar defines type of the nucleic acid. There are two classes of nucleic acids, called **deoxyribonucleic acid** (**DNA**) and **ribonucleic acid** (**RNA**). As the name suggests, **deoxyribonucleic acid** contains **deoxyribose**, whereas **ribonucleic acid** contains **ribose**.

Ribose

Ribose has three –OH groups, one each at carbon atoms 1, 2 and 3 of the pentose sugar.

Deoxyribose

Deoxyribose does not contain oxygen at the second carbon, and, thus, it has only two –OH groups (one each at carbon numbers 1 and 3). Deoxyribose thus, is also called 2-deoxyribose (Fig. 32.8).

The molecule of sugar is linked to the **nitrogenous base** on one side through **carbon number one** (**C-1**) and to **phosphoric acid** on the other side, through carbon number five (**C-5**).

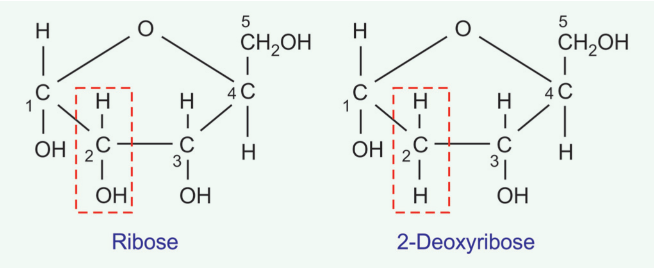


Fig. 32.8: Ribose and deoxyribose

NUCLEOSIDES

A nucleoside has two components and consists of a **nitrogenous base** and a **pentose sugar**.

The molecule of sugar (ribose or deoxyribose) is linked to the nitrogenous base with the N-glycosidic linkage.

To differentiate the carbon of the sugar from the carbon of the nitrogenous base, position of the **carbon of the sugar molecule** is shown with a **prime sign** (′), such as C-1′, C-5′, etc.

On the other hand, carbons and nitrogen of the nitrogenous bases are written without the prime sign, as **N-1**, **C-2**, etc.

Generally, carbon at the first position of the sugar, i.e. C-1′, is linked to N-1 of a pyrimidine or N-9 of a purine, i.e.

Base-N (1/9) - C-1′-Sugar

According to the presence of the type of sugar, nucleosides are classified as ribonucleosides and deoxyribonucleosides.

Ribonucleosides

Nucleosides containing **ribose** are called **ribonucleosides**. Accordingly, ribonucleosides of adenine, guanine, cytosine and uracil are designated as **adenosine**, **guanosine**, **cytidine** and **uridine**, respectively (Fig. 32.9).

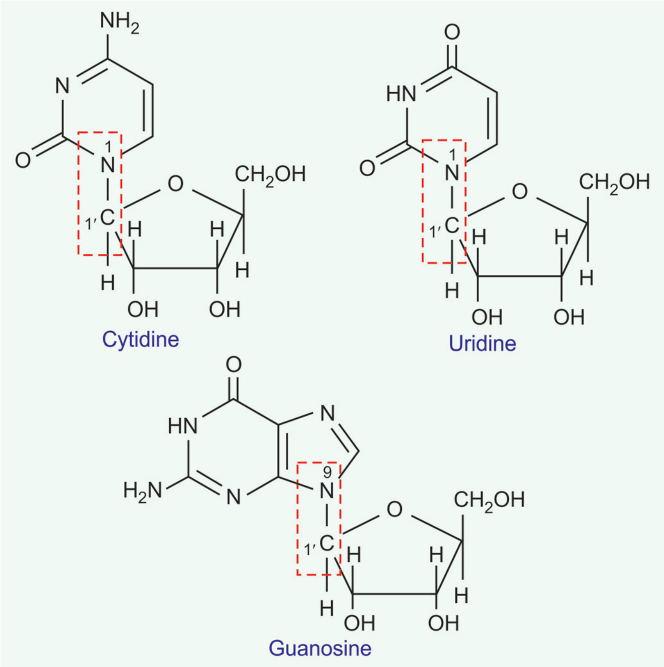


Fig. 32.9: Ribonucleosides

Deoxyribonucleosides

Nucleosides containing **deoxyribose** are designated as **deoxyribonucleosides**. These are written with a prefix deoxy- or d-. Thus, **deoxyadenosine**, **deoxyguanosine**, **deoxycytidine** and **deoxythymidine** are the nucleosides containing deoxyribose with adenine, guanine, cytosine and thymine, respectively. Since thymine is usually found in DNA only and is, generally, linked to deoxyribose, deoxythymidine can simply be referred to as **thymidine** (Fig. 32.10).

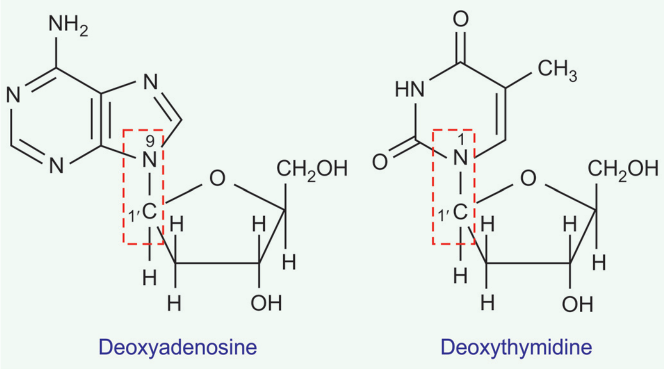


Fig. 32.10: Deoxyribonucleosides

NUCLEOTIDES

In addition to base and sugar (nucleoside), a nucleotide also has a molecule of phosphoric acid. The sugar molecule (ribose or deoxyribose) is, generally, linked to the nitrogenous base on one side and to phosphoric acid on the other side, e.g. **C-1′** of a **sugar** is linked to the **nitrogenous base** through **N-1 of a pyrimidine** or **N-9 of a purine**. Though any hydroxy group (–OH) of a sugar may be esterified with phosphoric acid, generally, C-5′ of a sugar is linked to phosphoric acid (also referred to as phosphate), i.e.

Base-N (1/9) - C-1′ -Sugar-C-5′-P

According to the presence of the type of sugar, nucleotides are classified as ribonucleotides and deoxyribonucleotides.

Ribonucleotides

Nucleotides of **ribose** are called **ribonucleotides**. Four ribonucleotides are generally obtained on hydrolysis of RNA. These are designated as **adenosine monophosphate** or **adenylate** (**AMP**), **guanylate** (**GMP**), **cytidylate** (**CMP**) and **uridylate** (**UMP**). Some of these are shown in Fig. 32.11.

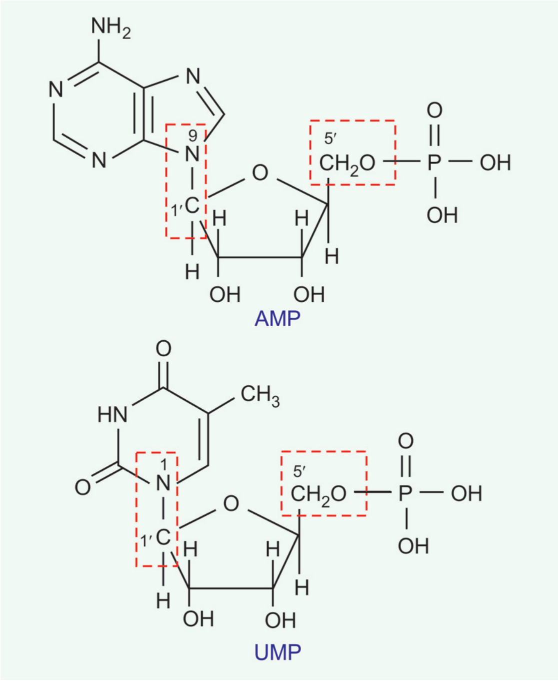


Fig. 32.11: Ribonucleotides

Deoxyribonucleotides

Deoxyribonucleotides are obtained on the hydrolysis of **DNA** and are designated as **dAMP**, **dGMP**, **dCMP** and **dTMP** (Fig. 32.12).

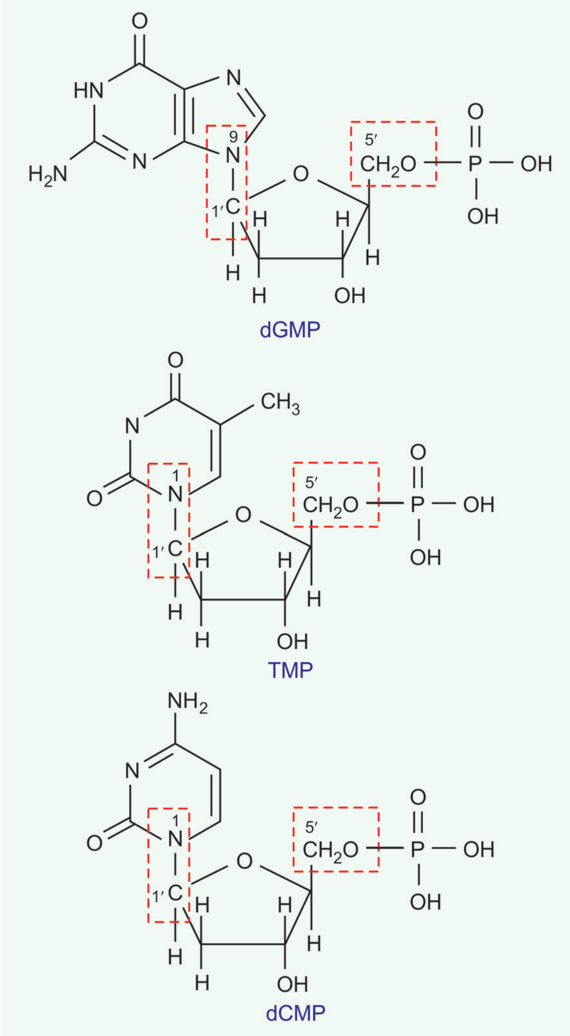


Fig. 32.12: Deoxyribonucleotides

Nitrogenous bases, **nucleosides** and **nucleotides**, which are commonly obtained on the hydrolysis of nucleic acids, are given in Table 32.2.

Table 32.2 Various nitrogenous bases, and the corresponding nucleosides and nucleotides found in RNA and DNA

Nitrogenous bases	Nucleosides (Base + Sugar)	Nucleotides (Base + Sugar + P)
RNA		
Adenine	Adenosine	AMP
Guanine	Guanosine	GMP
Cytosine	Cytidine	CMP
Uracil	Uridine	UMP
DNA		
Adenine	Deoxyadenosine	dAMP
Guanine	Deoxyguanosine	dGMP
Cytosine	Deoxycytidine	dCMP
Thymine	Thymidine	dTMP

Important Questions

1. Describe various features of Watson-Crick model of DNA.
2. Describe various types of RNA with their functions.
3. What is a nucleotide? Name different nucleotides found in DNA.
4. Write notes on:
    i. Differences between DNA and RNA
    ii. Various nitrogenous bases found in nucleic acids
    iii. B-DNA
    iv. A-DNA
    v. Z-DNA
    vi. Nucleosides
    vii. Nucleotides
    viii. hnRNA
    ix. Different types of DNA
    x. Structural features of tRNA
    xi. Messenger RNA

Chapter

33

Metabolism of Hemoglobin

Hemoglobin contains a prosthetic group called heme, which is attached to protein globin, a globular protein. It is found in red blood cells. Hemoglobin binds oxygen in the lungs and transports it to different parts of the body.

METABOLISM OF HEME

Synthesis of Heme

Heme consists of one atom of **ferrous iron** (Fe^{2+}) and a tetrapyrrole ring, called **protoporphyrin IX**. The ring is derived from eight residues of **glycine** and **succinyl CoA**. In the biosynthesis of heme, some of the reactions occur in mitochondria while others in the cytosol.

Outline of heme synthesis is given in Fig. 33.1.

1. In the first step, **glycine** condenses with **succinyl CoA** and forms **δ-aminolevulinic acid** (ALA), in the mitochondria. This reaction is catalyzed by the enzyme **δ-aminolevulinic acid synthase** (**ALA synthase**), which requires pyridoxalphosphate (PLP) as a coenzyme. This is the rate limiting step of heme synthesis.
2. In the next step, two molecules of **δ-aminolevulinic acid** condense, asymmetrically, and form **porphobilinogen**. This reaction is catalyzed by the enzyme **aminolevulinic acid dehydratase** (**ALA dehydratase**).

   ALA dehydratase is a zinc-containing enzyme, which occurs in cytosol. It consists of eight subunits, four of which interact with the substrate. ALA dehydratase is sensitive to heavy metals, particularly, lead. ALA levels increase in lead poisoning.
3. Thereafter, **porphobilinogen** undergoes deamination by the enzyme **porphobilinogen deaminase**, also called hydroxymethylbilane synthase or uroporphyrinogen I synthase, and is converted to a linear tetrapyrrole called **hydroxymethylbilane**.

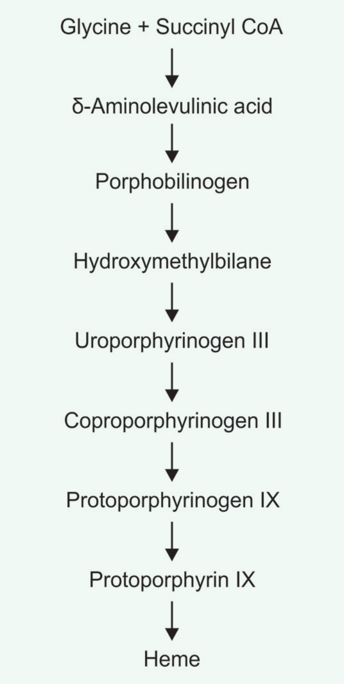


Fig. 33.1: Synthesis of heme

4. In the next step, **hydroxymethylbilane** is converted to **uroporphyrinogen I**, by ring closure, spontaneously.

   Alternatively, uroporphyrinogen III synthase **converts** hydroxymethylbilane to uroporphyrinogen III. Only, type I and type III isomers are found in nature.

   Porphyrins having **symmetric** arrangement of the substituent groups on the four pyrrole rings are designated as **type I** while those having **asymmetric** substitutions are referred to as **type III** (Fig. 33.2).

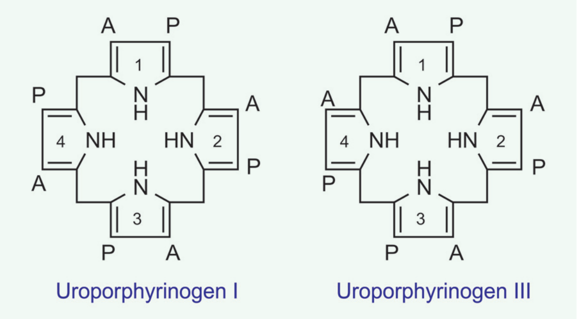


Fig. 33.2: Porphyrins: Symmetric and asymmetric substitutions

Under normal conditions, uroporphyrinogen formed is, almost, exclusively, type III. Heme is also a type III porphyrin.

5. In the next step, **uroporphyrinogen I/III** are converted to the respective **coproporphyrinogen** by the enzyme **uroporphyrinogen decarboxylase**. Iron salts inhibit uroporphyrinogen decarboxylase.
6. In the next reaction, **coproporphyrinogen III** enters the mitochondria and is converted to **protoporphyrinogen IX**. This reaction is catalyzed by the enzyme **coproporphyrinogen oxidase**, which requires molecular oxygen. This enzyme does not act on coproporphyrinogen I.

   These porphyrins are identified as IX, since they were designated ninth, in a series of the postulated isomers.
7. **Protoporphyrinogen oxidase** acts on **protoporphyrinogen IX** and converts it to **protoporphyrin IX**, in the mitochondria.

   Protoporphyrin IX is water insoluble. Its excessive amounts are excreted by the biliary system into the intestinal tract.
8. In the final step of heme synthesis, **ferrous iron** (Fe^{2+}) is incorporated into **protoporphyrin IX**. This reaction is catalyzed by the enzyme **ferrochelatase**, which requires some reducing substances. It is sensitive to heavy metals, especially, lead. It is also sensitive to iron deprivation.

PORPHYRIAS

Porphyrias are a group of disorders **due to abnormality in the pathway of heme biosynthesis**. It may be genetic or acquired.

Some types of porphyrias (**cutaneous porphyrias**) primarily affect the skin (Fig. 33.3).

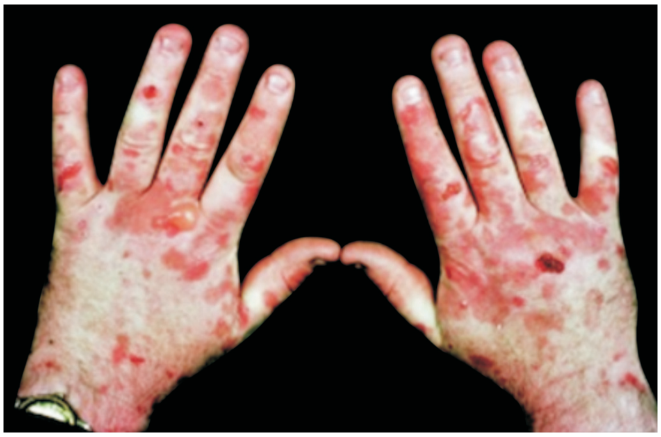


Fig. 33.3: Cutaneous porphyria

Areas of skin exposed to the sun become fragile and blistered, which can lead to infection, scarring, changes in skin color (pigmentation) and increased hair growth. Cutaneous porphyrias, include congenital erythropoietic porphyria, erythropoietic protoporphyria, hepatoerythropoietic porphyria and porphyria cutanea tarda.

Other types of porphyrias, primarily, affect the nervous system. These disorders are described as acute because their signs and symptoms appear quickly and, usually, last for a short time. Episodes of acute porphyria can cause abdominal pain, vomiting, constipation and diarrhea. During an episode, a person may also experience muscle weakness, seizures, fever and mental changes, such as anxiety and hallucinations.

Classification of Porphyrias

There are several types of porphyrias, which are distinguished by their genetic causes, signs and symptoms (Table 33.1).

1. **Acute intermittent porphyria:** It is due to **decreased activity of porphobilinogen deaminase**, alongwith the **increased activity of ALA synthase**.
2. **Erythropoietic porphyria:** It is due to abnormality in the **reticulocyte uroporphyrinogen III synthase**. It is associated with marked cutaneous light sensitization, and increased synthesis of uroporphyrinogen I and coproporphyrinogen I in the bone marrow.
3. **Porphyria cutanea tarda:** It is due to genetic deficiency of **uroporphyrinogen decarboxylase**. It shows cutaneous manifestations, primarily, sensitivity to light. The manifestation is expressed in patients who take drugs that cause an increase in porphyrin synthesis, or in individuals who consume large amount of alcohol. This, in turn, leads to

Table 33.1 Classification of porphyrias

Class of porphyria	Enzyme defect	Major symptoms	Laboratory findings
Acute intermittent porphyria	Increased ALA synthase, decreased porphobilinogen deaminase	Abdominal pain, neuropsychiatric symptoms	Urinary porphobilinogen (+)
Congenital erythropoietic porphyria	Decreased uroporphyrinogen III synthase	Photosensitivity	Urinary, faecal and red cell uroporphyrin I (+)
Porphyria cutanea tarda	Decreased uroporphyrinogen decarboxylase	Photosensitivity	Uroporphyrin (+)
Hereditary coproporphyria	Decreased coproporphyrinogen oxidase	Photosensitivity, abdominal pain, neuropsychiatric symptoms	Urinary porphobilinogen (+), urinary uroporphyrin (+), faecal protoporphyrin (+)
Variegate porphyria	Decreased protoporphyrinogen oxidase	Photosensitivity, abdominal pain, neuropsychiatric symptoms	Urinary porphobilinogen (+), faecal protoporphyrin (+)
Hereditary protoporphyria	Decreased ferrochelatase	Photosensitivity	Faecal protoporphyrin (+), red cell protoporphyrin (+)

accumulation of iron, which further inhibits uroporphyrinogen decarboxylase.

4. **Hereditary coproporphyria:** It is due to deficiency of the enzyme **coproporphyrinogen oxidase**.
5. **Variegate porphyria:** It is due to deficiency of the enzyme **protoporphyrinogen oxidase**.
6. **Hereditary protoporphyria:** It is due to inherited deficiency of the enzyme **ferrochelatase**.

DEGRADATION OF HEME

Heme is produced as a breakdown product of hemoglobin, which is released after hemolysis of the red blood cells.

Outline of heme degradation is given in Fig. 33.4.

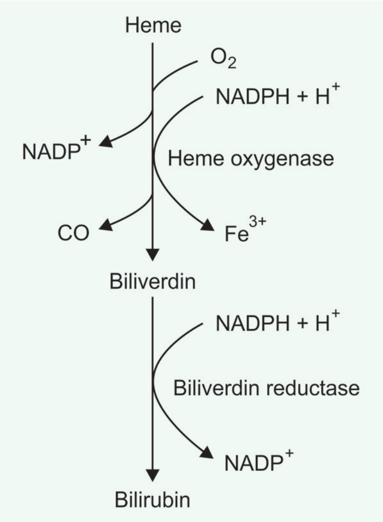


Fig. 33.4: Degradation of heme to bilirubin

- In the first step, heme is degraded in the reticuloendothelial cells to a linear tetrapyrrole called **biliverdin** IXα, by the microsomal enzyme system, designated as **heme oxygenase**. It requires molecular oxygen and NADPH, and is induced by heme. It catalyzes cleavage of the α-methenyl bridge, which is quantitatively converted to **carbon monoxide** (CO). CO is trapped by hemoglobin and exhaled.
- In the next step, **biliverdin** is reduced to **bilirubin** by the enzyme **biliverdin reductase**.

TYPES OF HEMOGLOBIN

Normal Hemoglobins

Different types of normal hemoglobins, found at various developmental stages in human, are shown in Table 33.2.

Table 33.2 Different types of human hemoglobins

Hemoglobin	Chain composition	Developmental stage
HbA_1	$\alpha_2\beta_2$	Adult
HbA_2	$\alpha_2\delta_2$	Adult
HbF	$\alpha_2\gamma_2$	Fetus
Hb·Gower-1	$\zeta_2\varepsilon_2$	Embryo
Hb·Portland	$\zeta_2\gamma_2$	Embryo

- **Embryonic hemoglobin:** Two forms of hemoglobins appear in the embryo, i.e. in the first month after conception. They contain two zeta chains alongwith either two epsilon chains ($\zeta_2\varepsilon_2$) or two gamma chains ($\zeta_2\gamma_2$). These are called Hb•Gower-1 and Hb•Portland, respectively.

- **Fetal hemoglobin:** This is the fetal form of hemoglobin and is designated as HbF. It predominates in the blood of the human fetus. HbF contains two α chains and two γ chains ($\alpha_2\gamma_2$). After birth, fetal hemoglobin is rapidly replaced with adult hemoglobin, whose synthesis actually starts in late fetal life.
- **Adult hemoglobin:** This is the major form of hemoglobin present in adults. It consists of two α chains and two β chains ($\alpha_2\beta_2$). This is also designated as HbA_1.
- **Minor adult hemoglobin:** It is also designated as HbA_2. It comprises about 2% of the normal adult hemoglobin and consists of two α chains and two delta chains ($\alpha_2\delta_2$).

Abnormal Hemoglobins

Mutant hemoglobins are called abnormal hemoglobins. Mutations cause instability in hemoglobin structure, increase or decrease O_2 affinity, or increase in rate of oxidation of heme iron from Fe^{2+} to Fe^{3+}.

Abnormal hemoglobin may arise by substitution of one amino acid by another, or by substitution of a peptide chain by another, which may be either too large or too small (to make the appropriate contacts), or by other means. These hemoglobins denature easily and precipitate within erythrocytes, forming Heinz bodies, which can damage erythrocyte membrane.

Patients with unstable hemoglobins develop hemoglobinopathies with characteristic features such as anemia, reticulocytosis, splenomegaly, etc.

Hemoglobinopathies

This is a heterogenous group of single gene disorders, which results in the formation of **structurally abnormal hemoglobin**. A mutant gene may result in substitution of one amino acid, in one type of the polypeptide chain, by another amino acid, or may result in loss of one or more amino acid residues in a polypeptide chain. In some cases, change may be clinically insignificant while in others, it may cause a serious disease.

Methemoglobinemia

Methemoglobin (HbM) is a **nonfunctional hemoglobin with Fe^{3+}** instead of Fe^{2+}. Patients with high concentration of methemoglobin show cyanosis (bluish color of the skin).

Sickle cell hemoglobin

Most common hemoglobinopathy is sickle cell disease. It is characterized by the presence of **hemoglobin S** (HbS). It is a variant form of the normal adult hemoglobin (HbA_1) in which substitution occurs at the sixth position of the β-globin chain. Whereas in HbA_1 this position is occupied by glutamic acid, in HbS, it is substituted by valine (β6, Glu → Val). The molecular basis for this is a point mutation, i.e. a change in a single nucleotide within the codon from GAA (or GAG), which code for glutamic acid, to GUA (or GUG), which code for valine. Due to the presence of valine in place of glutamic acid, sickle cell hemoglobin has a lower negative charge than normal hemoglobin.

As a result of this substitution, HbS gets polymerized and precipitates within the red blood cells, particularly, in the deoxygenated state. This, in turn, makes red blood cells assume **sickle shape** instead of the normal biconcave shape. Such distorted RBCs lose their deformability, resulting in higher rate of hemolysis, particularly, while negotiating through microcirculation. This type of hemolytic anemia is designated as **sickle cell anemia**. Hemoglobin S, though binds and releases oxygen, but abnormally (Fig. 33.5).

Only homozygous individuals for HbS exhibit the disease. Those who are heterozygous for HbS do not exhibit symptoms of sickle cell anemia except under extreme conditions of hypoxia, due to the reason that such individuals have 50% HbA_{1C} and 50% HbS in their red blood cells. This is called sickle cell trait. About 1% of the American blacks are heterozygous for HbS. They also have resistance to the malaria parasite. HbS can be detected by gel electrophoresis or by recombinant DNA technique.

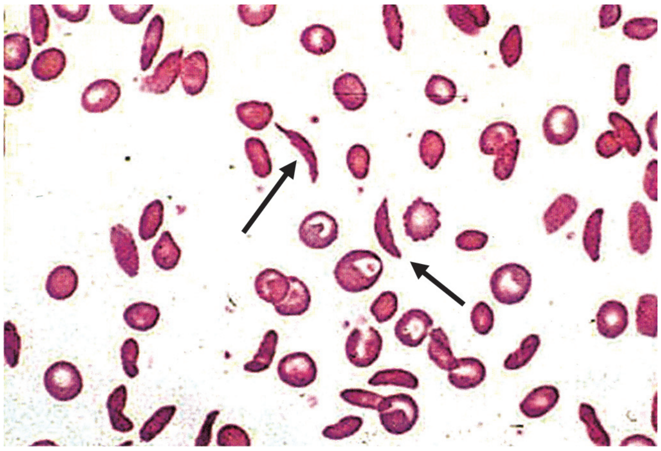


Fig. 33.5: Altered shape of RBCs in sickle cell anemia

Thalassemias

Thalassemias are a family of related genetic disorders that arise due to deletion of one or more globin-like genes in either the globin gene clusters, or due to a defect in transcription and/or processing of mRNA of the globin gene. If there is a reduced synthesis or total lack of synthesis of α-globin mRNA, the disease is classified as **α-thalassemia**. On the other hand, if β-globin mRNA level is affected, it is called **β-thalassemia**.

α-Thalassemia

α-Thalassemia patients may be missing one to four α-globin genes. If one α-globin gene is missing, the condition is called **α-thalassemia-1** (α-thal-1). When two α-globin genes are missing, it is called **α-thal-2**. Both the conditions are associated with mild to moderate anemia.

If three α-globin genes are missing, it results in the formation of a tetramer of four β-globins and this condition is called **HbH disease**.

When all the four α-globin genes are absent, it results in a fatal condition called **hydrops fetalis**.

β-Thalassemia

β-Thalassemias also exhibit different degree of severity and can be caused by a variety of defects or deletions.

Important Questions

1. Describe the process of heme synthesis.
2. What is porphyria? Describe various types of porphyrias.
3. Describe the process of heme degradation.
4. Write notes on:
    i. Sickle cell anemia
    ii. Hemoglobinopathies
    iii. Thalassemia

Chapter 34

Lipid Profile

For differentiation of disorders of lipoproteins, triglycerides and total cholesterol are routinely measured. The measurement of plasma total cholesterol is a first line screening test. **Most of the cholesterol present in plasma is found in LDL**. Similarly, **plasma triglycerides concentration** is a matter of the **sum total of VLDL and IDL.** To obtain a more accurate picture of lipid metabolism, we measure or calculate cholesterol present in the LDL and HDL fractions.

- The fasting lipid profile, i.e. the **concentration of total cholesterol, LDL-cholesterol, HDL-cholesterol and triglycerides,** provides information about the main **lipid markers of cardiovascular risk.**
- **LDL-cholesterol to HDL-cholesterol ratio** indicates the balance between cholesterol transport to and from peripheral tissues. A **ratio above 5 indicates increased cardiovascular risk**.
- Dividing the value of triglycerides by 5 gives the concentration of VLDL-cholesterol in plasma.

  VLDL-Cholesterol (mg/dL) = Triglycerides/5
- LDL-cholesterol concentration in plasma can also be calculated from the value of total cholesterol, triglycerides and HDL-cholesterol, by the equation, called Friedwald equation, as follows.

  LDL-Cholesterol (mg/dL) = Total cholesterol – [HDL cholesterol + Triglycerides/5]
- In about 95% of the patients with **primary hypercholesterolemia**, **plasma cholesterol concentration is** usually, **raised**, as the abnormality is due to a combination of dietary factors and a number of genetic abnormalities in handling cholesterol.
- **In patients with familial hypertriglyceridemia, plasma triglycerides (VLDL) concentration is increased** since this condition is associated with defects either in the production or in the catabolism of VLDL.
- **In familial combined hyperlipidemia,** there may be increase **in plasma LDL only, plasma VLDL only, or both.**

Patient Preparation

Serum lipids and lipoproteins are **measured after 10 hours of fast** because chylomicrons normally may be present in plasma up to 10 hours after a meal. Serum begins to appear hazy when the level of triglycerides exceeds above 200 mg/dL. Chylomicrons can be readily detected because they form a white supernatant layer, after refrigeration.

- Water is permitted during fasting.
- The patient should be on a normal diet, at least 7 days before the test.
- Lipid lowering drugs, estrogen, oral contraceptives should be stopped before the test.

Sample

Serum, heparinized plasma or EDTA plasma.

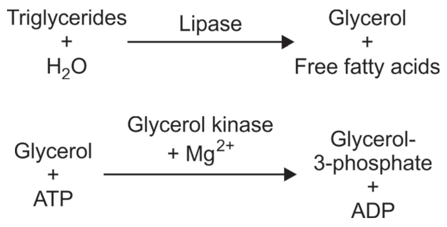
TOTAL CHOLESTEROL ESTIMATION

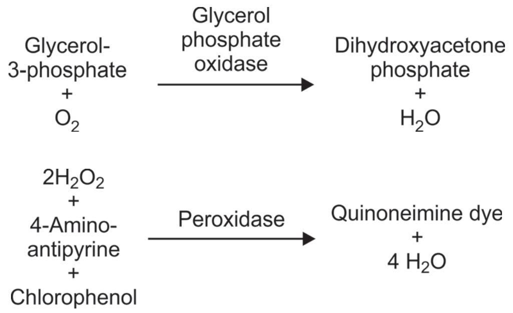
Estimation of total cholesterol has been discussed in Chapter 23.

ESTIMATION OF TRIGLYCERIDES

1. Enzymatic Method

Principle

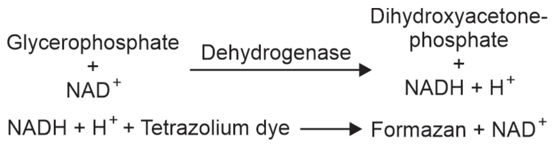




$$\text{Glycerol-3-phosphate} + O_2 \xrightarrow{\text{Glycerol phosphate oxidase}} \text{Dihydroxyacetone phosphate} + H_2O$$

$$2H_2O_2 + \text{4-Amino-antipyrine} + \text{Chlorophenol} \xrightarrow{\text{Peroxidase}} \text{Quinoneimine dye} + 4\,H_2O$$

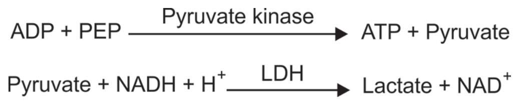
Alternatively, glycerophosphate is measured in a reduced form of NADH producing reaction and NADH is then measured spectrophotometrically at 340 nm.



$$\text{Glycerophosphate} + NAD^+ \xrightarrow{\text{Dehydrogenase}} \text{Dihydroxyacetone-phosphate} + NADH + H^+$$

$$NADH + H^+ + \text{Tetrazolium dye} \longrightarrow \text{Formazan} + NAD^+$$

Other method measures the ADP produced in reaction as shown in equation.



$$\text{ADP} + \text{PEP} \xrightarrow{\text{Pyruvate kinase}} \text{ATP} + \text{Pyruvate}$$

$$\text{Pyruvate} + NADH + H^+ \xrightarrow{\text{LDH}} \text{Lactate} + NAD^+$$

- The loss of NADH is measured at 340 nm.
- Enzymatic method for TG is specific in that they do not detect glucose or phospholipids.
- Linear up to 700 mg/dL.

Disadvantage

Free amount of glycerol in the sample is also measured [triglycerides (TG) blank is used].

TG blank

Endogenous glycerol concentration (1 mg/dL) contributes little to TG concentration (5–10 mg/dL), which is medically insignificant.

Blank is higher in certain conditions like –

1. Hypertriglyceridemia (> 200 mg/dL)
2. Diabetes mellitus (DM)
3. Total parenteral nutrition (TPN)
4. Hyperglycerolemia (deficiency of glycerokinase)

In these conditions TG blank is also measured and then it is subtracted from the total TG value.

Expected Values

• Normal	< 150 mg/dL
• Borderline high	150–199 mg/dL
• High	200–499 mg/dL
• Very high	≥500 mg/dL

ESTIMATION OF HDL

Reference Method

1. **Ultracentrifugation:** VLDL and chylomicrons are removed from sample by ultracentrifuging at 1,05,000 × *g* for 18 hrs. They accumulate as floating layer at the top of ultracentrifuge tube which is then removed by tube-slicing technique.
   Infranatant contains IDL, LDL, LP(a), HDL.
2. **Polyanion precipitation:** The apo B 100 containing lipids in this fraction are then precipitated with heparin sulfate and $MnCl_2$.
   The precipitate is then removed by centrifugation and cholesterol, in the clear supernatant, is measured.
   - As HDL has density range = 1.063–1.21 g/mL
   - LP(a) has density range = 1.04–1.08 g/mL, which overlaps with HDL-C. Therefore, precipitation step is used to separate HDL, to prevent its overestimation.

Methods for Separation of HDL

Precipitation (1st generation)

- Heparin-Mn^{2+}
- Dextran sulfate Mg^{2+}
- Phosphotungstate-Mg^{2+}

Precipitation methods (1st generation)

- Polyanion reacts with positively charged groups on lipoproteins; their action is facilitated in the presence of divalent cations which interacts with negatively charged groups causing aggregation and a cloudy precipitate.
- Precipitability depends on the lipid and protein compositions of the molecule. The greater the compositional differences, the more easily the lipoproteins are separated.

Sources of error

- Various precipitants differ in their abilities to precipitate apo B 100 containing lipids completely while leaving most species of HDL in solution, resulting in biases between precipitation methods.
- Hypertriglyceridemia (generally > 400 mg/dL), producing turbid supernatant or a floating layer over supernatant, produces significant error in HDL-C measurement.
- Matrix effect—due to addition of anticoagulants or preservatives.

Various steps to overcome these errors

- Sample can be ultracentrifuged and TG rich lipid be removed before precipitation.

- Centrifuging for a longer time or at greater g force.
- Sample can be diluted to decrease the concentration of TG rich lipoprotein. Dilution should not be greater than 2 fold, to prevent dilution errors.
- Pass the turbid supernatant through 0.45-µm filter to remove the unsedimented precipitation.

Facilitated separation (2nd generation)

- Magnetic with dextran sulfate-Mg^{2+}

Second generation methods

- Dextran sulfate precipitant is complexed with magnetic particles, allowing the lp-precipitant-magnetic particle complex to be removed rapidly by placing the reaction vessel on a magnetic disk.

Advantage

It reduces the interference associated with raised TG.

Homogenous assays (3rd generation)

- **1st homogeneous assay:** Requires 4 successive reagent addition:
  - Polyethylene glycol – causes aggregation of the apo B-100 containing chylomicrons, VLDL and LDL.
  - Antibodies to apo B 100 and apo C – protect the aggregated lipoproteins.
  - Cholesterol reaction enzymes – which act upon only the unprotected HDL-C. These include cholesterol esterase (CE), cholesterol oxidase (CO) and cholesterol peroxidase (CPO).
  - Guanidine salts – stop the colour reaction and solubilise the aggregate with guanidine salts, clearing the reaction mixture for measurement of colour.
- **2nd homogeneous assay:** Requires 2 reagents:
  - Sulfated alpha-dextrins with Mg^{2+} – selectively blocks the chylomicrons and VLDL without precipitation.
  - Polyethylene glycol – enhances the specificity of the enzymes CE and CO towards the cholesterol in HDL.
- **3rd homogenous assay:** It also includes 2 reagents:
  - Polyanion and polymer blocking agents – to block the non-HDL lipoproteins.
  - Detergents, enzymes and substrates – detergent exposes only cholesterol in HDL to the enzymes, giving specificity for HDL-C.
- **4th homogeneous assay:** It is based on immunoinhibition and includes 2 reagents:
  - Antibodies to human apo B 100 – blocks apo B 100-containing lipoproteins.
- **5th homogeneous assay:** This method allows cholesterol esterase and cholesterol oxidase to react with lipoproteins other than HDL, generating peroxides which, in turn, is scavanged by the enzyme catalase.

  An inhibitor of catalase and a surfactant, in a second reagent, specifically, reacts with HDL-C.

Interference

TG do not interfere up to 900 mg/dL regardless of whether the TG are in chylomicrons or VLDL.

- Studies showed LDL-C concentration upto 600 mg/L do not cause a significant bias, whereas LDL-C concentrations > 600 mg/L produce falsely increased HDL-C values.
- Bilirubin concentration below 10 mg/dL do not interfere with any of the homogeneous assays.
- Increased free fatty acids do not interfere, whereas paraproteinemias give discrepant results in some samples.
- Heparin, as anticoagulant, does not interfere, but results with EDTA plasma should be multiplied by 1.06 to compensate for dilution caused by osmotic shifting of water from the red cells.

Advantages

- The new homogeneous assays for HDL-C represent a technological breakthrough in achieving full automation of previously highly tedious manual pre-treatment methods.
- The homogeneous assays reduce operating costs while improving workflow and making the results more likely to conform to NCEP targets for analytical performance.
- The assays appear reasonably specific and free from the effects of major endogenous interferences.

Expected Values

- <40 mg/dL Low
- ≥60 mg/dL High

ESTIMATION OF LDL

Reference Method

It is measured by the same method as HDL-cholesterol. After ultracentrifugation, bottom fraction is subjected to precipitation by heparin and manganese.

- LDL-C is measured by difference between cholesterol in supernatant and cholesterol in d>1.006 fraction.

- This measured fraction of LDL is known as **broad-cut** fraction because it includes IDL and Lp(a).

Indirect Methods

This includes Friedwald equation

- LDL-C = [TOTAL-C] – [HDL-C] – [TG/5]
- TG/5 is an estimate of VLDL concentration and is based on average ratio of TG to cholesterol in VLDL.
- All concentrations are in mg/dL.
- If LDL is expressed in mmol/L then the equation will be:

  LDL-C = [TOTAL-C] – [HDL-C] – [TG/2.22]

Conditions Where this Equation Cannot be Used

1. TG > 400 mg/dL (error > 10%).
2. Sample with raised chylomicron level (non-fasting).
3. Type 3 hyperlipoproteinemia, which is characterised by presence of beta-VLDL (much richer in cholesterol than normal VLDL). So, TG/cholesterol ratio of 3 : 1, should be used, as TG/5 factor in equation would underestimate VLDL-C and, in turn, overestimate LDL-C.

Direct Methods

It includes:

1. Selective precipitation
2. Homogeneous assays

1. Selective Precipitation

Several direct methods have been used for LDL-C measurement that are based on selective precipitation with polyvinyl sulfate or heparin at low pH. LDL-C is then calculated as the difference between total cholesterol and that in the supernatant.

A more specific method uses a mixture of polyclonal antibodies to apo A1 and apo E, linked to a resin, to bind VLDL, IDL and HDL, with LDL-C measured in a filtrate by the usual methods.

2. Homogeneous Assays

Homogeneous LDL-cholesterol methods allow convenient determination of LDL-cholesterol values in a small sample volume on an automated analyzer without any sample pretreatment.

5 homogeneous LDL methods are commercially available.

- **1st homogeneous assay:** In this, LDL is directly measured by suppressing other lipoproteins. It includes 2 reagents:
  1. First reagent consists of $MgCl_2$, dye, buffer and the alpha-cyclodextrin sulfate, which has a highly concentrated negative charge to mask cholesterol in chylomicrons and VLDL, in the presence of Mg^{2+}.
  2. Second reagent includes enzymes cholesterol oxidase (CO), cholesterol esterase (CE) and cholesterol peroxidase (CPO), dye, buffer (pH 6.75) and a polyoxyethylene-polyoxypropylene polyether (POE-POP) to block cholesterol, especially, in HDL.
- **2nd homogeneous method:** It also includes 2 reagents:
  1. The first reagent contains ascorbic acid oxidase, 4-aminoantipyrene, peroxidase, CO, CE, buffer (pH 6.3) and a detergent, which solubilises all non-LDL lipoproteins, allowing reaction of their cholesterol with the esterase and oxidase ezymes, forming a colourless product.
  2. The second reagent contains N,N-bis-(4-sulfobutyl)-m-toluidine·Na_2 (DSBmT), buffer (pH 6.3) and a detergent to specifically release LDL cholesterol.

     The resulting H_2O_2 reacts with N,N′-bis-(4-sulfobutyl)-M-toluidine disodium salt to generate a colored product.
- **3rd homogeneous method:** This method uses an enzymatic complex to stabilize LDL molecules, so the cholesterol is nonreactive. Cholesterol from non-LDL lipoproteins reacts with cholesterol esterase (CE), cholesterol oxidase (CO), catalase and *N*-(2-hydroxy-3-sulfopropyl)-3,5-dimethoxyaniline to produce colorless products.

  A second reagent is added that contains sodium azide to inhibit catalase and a detergent that releases cholesterol from LDL. The cholesterol reacts with components of reagent 1 plus peroxidase and 4-aminoantipyrene in reagent 2, to form a colored product that is measured spectrophotometrically.
- **4th homogeneous method:** In this method non-LDL-C is removed via a selective reaction with CO and CE with the resulting peroxide byproduct eliminated by reaction with catalase.

  First reagent contains $MgCl_2$, CO, CE, catalase and N-(2-hydroxy-3-sulfopropyl)-3,5-dimethoxyaniline sodium salt, Emulgen 66 and Emulgen 90 in Good's buffer.

  Second reagent contains peroxidase, 4-aminoantipyrine, sodium azide and Triton X-100 in Good's buffer.

- **5th homogeneous method:** First reagent contains the detergent calixarene, that converts LDL to a soluble complex. Cholesterol esters of HDL-C and VLDL-C are preferentially hydrolysed by CE, CO and hydrazine, which divert the accessible cholesterol to cholestenone hydrazone.

  Second reagent contains deoxycholate which breaks up the LDL-caliaxrene complex, allowing LDL-C to react with esterase, dehydrogenase and beta-NAD, to yield cholestenone and beta-NADH, which is then measured by spectrophotometer.

ESTIMATION OF VLDL

It is measured indirectly by the Friedwald equation (if cholesterol in blood is less than 400 mg/dL):

$$\text{VLDL} = \text{TG}/5$$

ESTIMATION OF TOTAL LIPIDS

Total lipids can be estimated by sulfophosphovanillin method.

Principle

Lipids react with vanillin in presence of sulfuric and phosphoric acids to form a pink colored complex.

Reagents

1. Total lipid standard, 1000 mg/dL
2. Color reagent (phosphovanillin): It is prepared by mixing:
   a. 0.61 g/dL vanillin, 350 mL
   b. Orthophosphoric acid, 600 mL
   c. Distilled water, 50 mL
3. Concentrate sulfuric acid

Procedure

Take two test tubes and mark them as test and standard. Add standard solution, serum and concentrate sulphuric acid as shown in Table 34.1.

Table 34.1 Reaction of the serum and the standard solution with sulphuric acid

	Test	Standard
Standard	—	0.05 mL
Serum	0.05 mL	—
Conc sulfuric acid	2 mL	2 mL

Mix and keep in boiling water bath for 10 min after plugging the tubes with wool.

After 10 min, contents of the above tubes are used for the estimation of total lipids. For this purpose, take three test tubes and mark them as test, standard and blank. Add various solutions as shown in Table 34.2.

Table 34.2 Preparation of the test tubes for the estimation of total lipids in serum

	Test	Standard	Blank
From above tubes	0.10 mL	0.10 mL	—
Conc sulfuric acid	—	—	0.10 mL
Color reagent	2.5 mL	2.5 mL	2.5 mL

Mix and keep at room temperature for 15 min. Read absorbance of test and standard against blank at 540 nm.

Calculations

$$\text{Serum total lipids, mg/dL} = \frac{\text{OD}_{\text{test}}}{\text{OD}_{\text{standard}}} \times 1000$$

Normal Values

400–1000 mg/dL

PRACTICAL EXERCISE

1. Estimate various parameters of lipid profile and interpret your result.

Important Question

1. What do you mean by lipid profile? What is its significance?

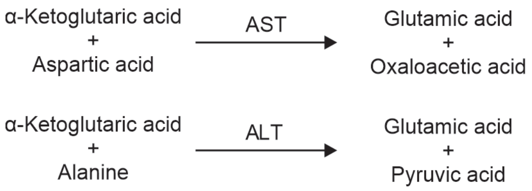
Chapter

35

Estimation of Serum Enzymes

SERUM TRANSAMINASES (SGOT/SGPT)

Serum glutamate oxaloacetate transaminase (**SGOT**), also known as aspartate aminotransferase (AST) and serum glutamate pyruvate transaminase (**SGPT**), also called alanine aminotransferase (ALT), catalyse the following reactions, respectively.



$$\begin{matrix}\text{α-Ketoglutaric acid} \\ + \\ \text{Aspartic acid}\end{matrix} \xrightarrow{\text{AST}} \begin{matrix}\text{Glutamic acid} \\ + \\ \text{Oxaloacetic acid}\end{matrix}$$

$$\begin{matrix}\text{α-Ketoglutaric acid} \\ + \\ \text{Alanine}\end{matrix} \xrightarrow{\text{ALT}} \begin{matrix}\text{Glutamic acid} \\ + \\ \text{Pyruvic acid}\end{matrix}$$

AST or ALT activity can be measured either colorimetrically or spectrophotometrically.

Colorimetric Method

Principle

The reactions catalysed by the two transaminases, as described above, take place by adding α-ketoglutaric acid and aspartic acid for AST, or α-ketoglutaric acid and alanine for ALT. The reaction is stopped by dinitrophenylhydrazine (DNPH) reagent. DNPH reacts with pyruvate to form a brown colored complex in alkaline medium, which is measured colorimetrically.

Since the final product formed in both the reactions is the pyruvic acid (because the oxaloacetate formed in the AST-catalysed reaction also spontaneously gets converted to pyruvic acid), accordingly the standard solution, used in the procedure, contains pyruvate only.

Reagents

1. Buffered substrate: Dissolve 15 g of K_2HPO_4 and 2 g of KH_2PO_4 (phosphate buffer, 100 mM/L) and 300 mg of α-ketoglutaric acid in 800 mL of water. Adjust the pH to 7.4 with sodium hydroxide solution. Thereafter, add 13.2 g of aspartic acid (for AST), or 17.8 g of D-alanine (for ALT), and make the volume to 1 litre with water.
2. DNPH reagent (1 mmol/L): 200 mg of dinitrophenylhydrazine in 1 mol/L HCl.
3. Sodium hydroxide solution (0.4 M).
4. Standard pyruvate solution—2 mmol/L.

Procedure

Take four clean and dry test tubes, and label them as test (T), control (C), standard (S) and blank (B). Then add the following in each of test tubes, separately, as shown in Table 35.1.

Table 35.1 Preparation of the test tubes for the estimation of serum SGPT (ALT) and SGOT (AST) by colorimetric method

Contents	T	C	S	B
Buffered substrate (pH 7.4)	1.0 mL	1.0 mL	–	1.0 mL
Serum	0.2 mL	–	–	–
Standard pyruvate solution	–	–	0.2 mL	–
Distilled water	–	–	–	0.2 mL
Mix and incubate at 37°C for 30 min for ALT or 60 min for AST				
DNPH reagent	1.0 mL	1.0 mL	1.0 mL	1.0 mL
Buffered substrate	–	–	1.0 mL	–
Serum	–	0.2 mL	–	–
Mix and keep at room temperature for 20 min				
Sodium hydroxide solution	10 mL	10 mL	10 mL	10 mL

Mix and take OD at 520 nm after 5 min

Calculation

$$\text{ALT activity (Karman U/L)} = \frac{OD_T - OD_C}{OD_s - OD_B} \times \frac{0.4}{0.2} \times \frac{1000}{30}$$

$$= \frac{OD_T - OD_C}{OD_s - OD_B} \times 66.7$$

As 1 Karman U/L = 0.487 IU/L, therefore

$$\text{ALT (SGPT) activity (IU/L)} = \frac{OD_T - OD_C}{OD_s - OD_B} \times 66.7 \times 0.487$$

$$= \frac{OD_T - OD_C}{OD_s - OD_B} \times 32.48$$

Since for AST the incubation time is 60 min, therefore AST (SGOT) activity (IU/L)

$$= \frac{OD_T - OD_C}{OD_s - OD_B} \times \frac{0.4}{0.2} \times \frac{1000}{60} \times 0.487$$

$$= \frac{OD_T - OD_C}{OD_s - OD_B} \times 16.24$$

Spectrophotometric Method

Principle

Alanine aminotransferase catalyzes the following reaction:

$$\begin{matrix}\text{L-Alanine} \\ + \\ \text{2-Oxoglutarate}\end{matrix} \xrightarrow{\text{GPT}} \begin{matrix}\text{Pyruvate} \\ + \\ \text{Glutamate}\end{matrix}$$

The product (pyruvate), so formed oxidizes NADH in the coupled enzymatic reaction, as follows:

$$\text{Pyruvate} + \text{NADH} + \text{H}^+ \xrightarrow{\text{LDH}} \text{Lactate} + \text{NAD}^+$$

This reaction is followed spectrophotometrically, by measuring the decrease in absorbance due to utilization of NADH at 340 nm, with time.

Reagents

1. Phosphate (100 mM)-alanine (1 M) buffer, pH 7.4: Dissolve 0.2 g of KH_2PO_4, 1.5 g of K_2HPO_4, and 8.9 g of L-alanine in distilled water. Adjust the pH to 7.4 and make up to 100 mL. Add 0.3 mL of chloroform to prevent bacterial growth on storage.
2. NADH solution (13 mM): Dissolve 16 mg of NADH.Na_2 and 15 mg of $NaHCO_3$ in 1.5 mL of distilled water.
3. Lactate dehydrogenase (0.5 mg protein/mL): Dilute commercial LDH solution in glycerol (50% glycerol, v/v).
4. 2-Oxoglutarate (660 mM): Dissolve 290 mg of 2-oxoglutaric acid in approximately 2 mL of distilled water. Adjust the pH with 1 N NaOH, in an ice bath, and make up to 3 mL with water (375 mg of disodium salt of 2-oxoglutaric acid may also be directly dissolved in 3 mL of distilled water).

Procedure

Prepare a reagent mixture by mixing phosphate-alanine solution (30 volumes), NADH solution (0.5 volume) and LDH solution (0.5 volume). It is stable for 12 h at 25°C. Pipette into the cuvettes, 3.1 mL of the reagent mixture and 0.5 mL of the serum sample, mix, and incubate for 5 min at 25°C. Add 100 µL of 2-oxoglutarate solution, mix and follow the reaction at 25°C, at 340 nm for 5–10 min.

Calculation

Alanine aminotransferase activity (U/L) = 1190 A_{340} nm/min.

Reference Range

ALT (SGPT)	8–56 IU/L
AST (SGOT)	8–40 IU/L

ALKALINE PHOSPHATASE

King and Armstrong method

Principle

$$\begin{matrix}\text{p-Nitrophenylphosphate} \\ + \\ \text{H}_2\text{O}\end{matrix} \xrightarrow{\text{Alkaline phosphatase}} \begin{matrix}\text{Nitrophenol} \\ + \\ \text{H}_3\text{PO}_4\end{matrix}$$

The amount of p-nitrophenol liberated in unit time, as determined in alkaline pH (8.5), at 400–420 nm, is a measure of alkaline phosphatase activity.

Reagents

1. p-Nitrophenylphosphate buffer (buffered-substrate solution): Dissolve 1.052 g of diethanolamine, 8 mL of 0.1 N HCl, and 46.4 mg of p-nitrophenylphosphate in 85 mL of double distilled water. Adjust the pH to 9.8 and dilute to 100 mL with distilled water. It is stable for a week, at 0–4°C.

   (Alternatively, disodium phenylphosphate can also be used as a substrate.)
2. Serum: Fresh, nonhemolysed serum is used.
3. 0.05 N NaOH.

Procedure

Pipette 2.0 mL of buffer-substrate solution and 50 mL of serum. Mix and incubate for exactly 30 min at 25°C. Stop the reaction by adding 10 mL of 0.05 N NaOH. For blank, the sample is added after the addition of NaOH. Read the absorbance at 405 nm against the blank.

Calculation

$$\text{Alkaline phosphatase activity (U/L)} = \frac{\Delta A_{405}}{30} \times \frac{12.05 \times 1000}{18.5 \times 0.05}$$

$$= \Delta A_{405} \times 434$$

King and Armstrong Method Using 4-aminoantipyrine

Principle

Alkaline phosphatase hydrolyses disodium phenylphosphate in alkaline medium and liberates phenol, which gives a reddish to purple color in the presence of the alkaline oxidizing agent, 4-aminophenazone (4-aminoantipyrine). The color, thus, developed is stable for at least one hour in the absence of bright light.

Reagents

1. Substrate: Dissolve 2.18 g of disodium phenylphosphate (or 2.541 g of disodium phenylphosphate.tetrahydrate) in 1 litre of distilled water. Boil, cool and add few drops of chloroform.
2. Buffer: Dissolve 6.36 g of anhydrous sodium carbonate and 3.36 g of sodium bicarbonate in distilled water and make up the volume to 1 litre.
3. Buffered substrate: Mix equal volume of the substrate and the buffer, the pH of the solution should be 10.
4. Standard phenol solution:
   i. Stock standard solution: 1 g/L in 100 mM HCl.
   ii. Working standard solution: Dilute the stock standard 100 times to give 10 µg/mL.
5. Sodium hydroxide: 500 mmol/L.
6. 4-Aminophenazone (4-aminoantipyrine): 6 g/L in distilled water.
7. Potassium ferricyanide: 24 g/L in distilled water.

Procedure

Take four test tubes and mark as test (T), control (C), standard (S) and blank (B). Add various reagents as shown in Table 35.2.

Calculation

Since 1 KAU is equal to 7.1 IU/L, serum alkaline phosphatase (ALP) activity can be calculated as follows:

$$\text{Serum ALP (IU/L)} = \frac{OD_T - OD_C}{OD_S - OD_B} \times 10 \times 7.1$$

Reference Range

39 – 90 IU/L for adults

47 – 406 IU/L for pediatric age group

Table 35.2 Preparation of test tubes for the estimation of serum alkaline phosphatase by the King and Armstrong method using 4-aminoantipyrine

Contents	T	C	S	B
Buffer (pH 10.0)	1 mL	1 mL	1.1 mL	1.1 mL
Buffered substrate	1 mL	1 mL		
Mix and incubate at 37°C for 3 min				
Standard phenol (1mg/100 mL)	–	–	1 mL	–
Distilled water	–	–	–	1 mL
Serum	0.1 mL	–	–	–
Mix and incubate at 37°C for 15 min				
NaOH (0.5 N)	0.8 mL	0.8 mL	0.8 mL	0.8 mL
Serum	–	0.1 mL	–	–
$NaHCO_3$	1.2 mL	1.2 mL	1.2 mL	1.2 mL
4-Aminoantipyrine	1 mL	1 mL	1 mL	1 mL
Potassium ferricyanide	1 mL	1 mL	1 mL	1 mL

Mix thoroughly after the addition of each reagent and read OD at 520 nm.

Bodansky Method

Principle

In this method β-glycerophosphate is used as substrate and the inorganic phosphorus, liberated from the substrate, is estimated by Fiske and Subbarow method.

Reagents

1. Substrate: Into a 100 mL flask, add 3.6 mL of petroleum ether, 80 mL of distilled water, 0.5 g of sodium β-glycerophosphate and 0.424 g of sodium diethylbarbiturate. The volume is made up to 100 mL with water. The solution is adjusted to pH 8.5 and stored in cold.
2. 30% TCA.

Procedure

Substrate solution (0.9 mL) is measured into a centrifuge tube and placed in a water bath at 37°C for few min. Serum (0.1) mL is added to it, mixed the contents and incubate the tube for 1 hour. Add 0.2 mL of 30% TCA, centrifuge at 3000 rpm for 5 min and collect the supernatant. A blank tube is also run with serum in which the reaction is stopped at zero minute. Follow the procedure for phosphorus estimation as described by Fiske and Subbarow (Chapter 49).

The liberated phosphate can also be estimated by **Chen's method**, which is more sensitive, and is as follows:

Chen's Method

Reagent

Ammonium molybdate reagent: Mix 1 volume of 6 N H_2SO_4, 2 volumes of distilled water, 1 volume of 2.5% ammonium molybdate and 1 volume of 10% ascorbic acid.

Procedure

Take adequate volume of the supernatant and make up to 1.0 mL with deionized water. Add 2 mL of freshly prepared ammonium molybdate reagent. Mix the contents and incubate at 37°C for 90 min. The absorbance is recorded at 820 nm in a spectrophotometer.

Calculation

Alkaline phosphatase activity is expressed as μmoles of phosphorus liberated, as mg protein/min (or /h) per100 mL of serum at pH 8.5.

ACID PHOSPHATASE

Principle

It hydrolyzes ortho-phosphate ester at optimum pH of 4.9.

Reagents

1. Citrate buffer, pH 4.9: Dissolve 21 g of citric acid in 188 mL of 1 N NaOH, dilute to 500 mL with water and adjust the pH to 4.9.
2. Substrate: Disodiumphenylphosphate, 10 mM/L (2.8 g/L of water).
3. Buffered substrate: Prepare fresh by mixing equal volumes of 1 and 2.
4. Sodium hydroxide, 0.5 N.
5. Sodium bicarbonate, 0.3 N.
6. 4-Aminophenazone: 6 g/L in water.
7. Stock phenol standard: 1 g pure crystalline phenol/L.
8. Working standard: Dilute stock standard 1 : 10.

Procedure and Calculation

These are similar, as described, for alkaline phosphatase.

Reference Range

Up to 3 U/L

AMYLASE

Principle

The activity is measured by determining the amount of starch, it can split in a given time. The amount of starch in the reaction mixture is estimated by measuring the color it produces with a weak iodine solution.

Reagents

1. Standard starch solution: 0.8 g of starch and 0.1 g of benzoic acid are dissolved in boiling water, cooled and the volume is made up to 100 mL
2. Phosphate buffer (0.1 M, pH 7.4)
3. Gram Lugol's iodine solution: 1.0 g of iodine and 2.0 g of potassium iodide are dissolved in distilled water and the volume is made up to 100 mL.
4. 5% H_2SO_4

Procedure

To 0.5 mL of starch solution, in a large test tube, add 0.5 mL of the phosphate buffer. To this, add 0.2 mL of serum and the mixture is incubated at 37°C for 30 min. At the end of this period, add 2 mL of 5% H_2SO_4 and 1 mL of iodine solution. Make the volume to 50 mL with distilled water. A control tube is prepared by adding 5% H_2SO_4 immediately after the addition of serum and procedure is followed, similarly, as for the test. The color is measured at 640 nm against reagent blank, which is prepared either without starch or serum.

Calculation

$$\begin{array}{c}\text{Serum amylase}\\(\text{U}/\text{L})\end{array} = \frac{\text{OD}_{\text{Test}} - \text{OD}_{\text{Control}}}{\text{OD}_{\text{Standard}}} \times 4 \times \frac{10}{0.2}$$

Note: One unit of amylase activity is defined as the amount of starch, in mg, hydrolyzed by 10 mL of serum, in 30 min.

Reference Range

28–100 units/L for adults.

PRACTICAL EXERCISES

1. Estimate GOT and GPT levels in serum.
2. Estimate alkaline phosphatase activity in serum.
3. Estimate acid phosphatase activity in serum.
4. Estimate amylase activity in serum.

Important Questions

1. Describe reference range of SGOT/SGPT.
2. Describe reference range of alkaline phosphatase.
3. Describe reference range of acid phosphatase.
4. Describe reference range of amylase.

Chapter

36

Spectroscopy

Spectroscopy refers to the **study of wavelength-dependence of absorption and emission of the visible light dispersed by prism, by matter.** Depending upon the nature of interaction between the energy and the material, **spectroscopy may be of different types** such as atomic spectroscopy, absorption spectroscopy, emission spectroscopy, nuclear spectroscopy, etc.

Atomic spectroscopy was the first application of spectroscopy **involving visible light**. Accordingly, **when white light is dispersed by a prism, we see all colours (wavelengths) of the visible spectrum.** When **atoms are excited, they give-off (emit) light** and that **different elements produce light of different colours (wavelengths).**

For routine spectroscopy a **direct vision spectroscope** is generally used. It **consists of a cylindrical metallic tube containing a prism of crown glass** and the **other one of the flint glass** which are **placed together.** A **slit of variable aperture at one end of the instrument permits the light to enter**. An **eyepiece is placed at the other end** of the spectroscope **through which one can observe the spectrum.**

Principle of Direct Vision Spectroscope

When daylight is viewed through spectroscope, two dark lines are seen, normally called Fraunhofer's lines. They are at 589 and 518 nm, and arise due to absorption of light of particular wavelengths by sodium and magnesium present in the solar atmosphere (Fig. 36.1).

These dark lines are helpful in detecting the gross appearance of different types of hemoglobin (Fig. 36.2).

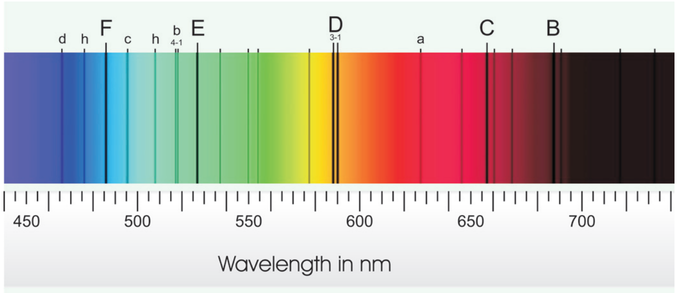


Fig. 36.1: Light spectrum and the Fraunhofer's lines seen through the spectroscope

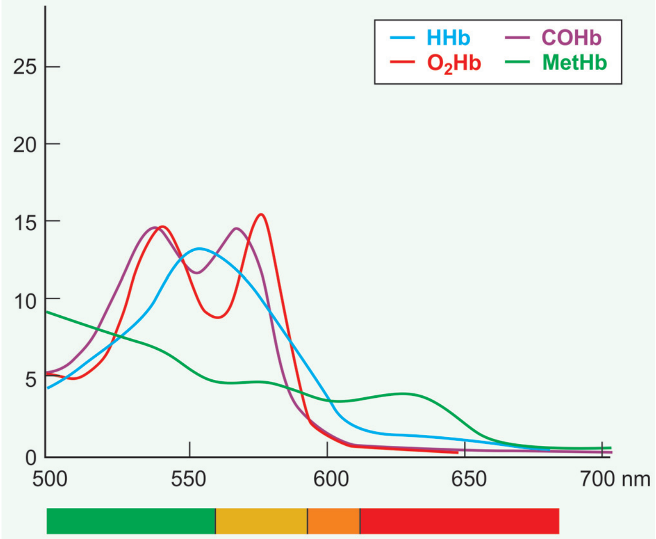


Fig. 36.2: Absorption maxima of different types of hemoglobin

OXYHEMOGLOBIN (HbO_2)

It is pale red in color, on naked eye examination.

Preparation

It is prepared by adding one drop of blood in 5 mL of water (1 : 200 dilution).

Observation

Two bands are seen in green portion of spectrum (539 nm and 577 nm), known as alpha and beta bands.

CARBOXYHEMOGLOBIN

This is formed on exposure of hemoglobin to carbon monoxide. It is cherry red in color.

Preparation

When diluted blood sample is subjected to either coal gas or a mixture of CO and CO_2, obtained by treating oxalic acid with concentrated H_2SO_4.

$$\text{Oxalic acid} \xrightarrow{\text{Conc. } H_2SO_4} CO_2 + CO + H_2O$$

Add a drop of caprylic alcohol (2-octanol), to prevent frothing during bubbling.

Observation

Two bands are seen in green spectrum at 572 and 534 nm.

Clinical Application

COHb is formed in various poisoning states:

1. In chronic smokers, carboxyhemoglobin is present
2. Vehicle exhaust poisoning
3. Coal mine workers
4. If a person is trapped in car with a running engine and due to burning of fuel he gets exposed to CO exhaust which can be fatal. This is because Hb has 200 times more affinity for carbon monoxide than oxygen, forming COHb that leads to inhibition of cytochrome oxidase of electron transport chain resulting in respiratory arrest.

Symptoms

Headache, dizziness, nausea, vomiting, unconsciousness, followed by death.

METHEMOGLOBIN

This is the oxidation product of hemoglobin in which Fe^{2+} is converted into Fe^{3+} form. It is chocolate brown in color.

Preparation

In 4 drops of blood, add 5 mL of water and a pinch of potassium ferricyanide $[K_3Fe(CN)_6]$ and mix gently. Solution turns brown.

Observation

A band is seen in red region at 638 nm. A very faint band may also be seen in the blue region on using a wider slit.

Clinical Application

Normal persons carry < 1% of metHb.

- Congenital methemoglobinemia: Enzyme metHb reductase is deficient for conversion of metHb to Hb.
- Certain drugs and chemicals, i.e. nitrobenzene, aniline, sulphonamides inhibit the enzyme leading to increased metHb.
- During cyanide poisoning, cyanide combines with Hb and produces lethal effects on the body but metHb combines with cyanide to form cyanmetHb which is excreted in urine and lethal effect of cyanide is reduced. $NaNO_2$ is given to convert Fe^{3+} to Fe^{2+}.

PRACTICAL EXERCISE

1. Examine the hemoglobin solutions for oxyhemoglobin, carboxyhemoglobin and methemoglobin.

Important Question

1. Write notes on:
   - i. Oxyhemoglobin
   - ii. Carboxyhemoglobin
   - iii. Methemoglobin
   - iv. Spectroscopy

Chapter

37

Chromatography

Chromatography is a technique that separates analytes based on their **differential distribution** between a **stationary** and a **mobile phase**. This differential distribution is on the basis of differences of size, shape, mass, charge and solubility, and adsorption properties of the molecules, to be separated.

Thus, three components form the basis of the chromatography techniques, i.e.:

1. **Stationary phase:** This phase is composed of a **solid phase** or **a layer of a liquid adsorbed on the surface a solid support**.
2. **Mobile phase:** This phase is composed of **liquid** or **a gaseous component**. If mobile phase is liquid, it is termed liquid chromatography (LC), and if it is gas, it is called gas chromatography (GC).
3. **Separated molecules**.

TYPES OF CHROMATOGRAPHY

Various chromatography methods include **adsorption chromatography, column chromatography, ion exchange chromatography, gel filtration chromatography, affinity chromatography, partition chromatography, paper chromatography, thin layer chromatography (TLC), gas chromatography** and **high pressure liquid chromatography**.

Column chromatography, like ion exchange chromatography and affinity chromatography, is more effective in the separation of macromolecules, such as nucleic acids and proteins. Agarose gel chromatography is used for the purification of RNA and DNA particles and viruses. Chromatographic methods, based on partition, are very effective on separation and identification of small molecules, as amino acids, carbohydrates and fatty acids. Paper chromatography is used in the separation of proteins and in studies related to protein synthesis. Gas–liquid chromatography is utilized in the separation of alcohol, ether, lipid and amino groups, and observation of enzymatic interactions.

ADSORPTION CHROMATOGRAPHY

Adsorption chromatography is one of the oldest types of chromatography, frequently referred to as **liquid–solid chromatography**. The technique was developed by Russian botanist Tswett, who first separated plant pigments and assigned the name chromatography. Now, even the colourless substances can be separated by chromatography.

Adsorption chromatography utilizes a **mobile liquid** or **gaseous phase,** which is adsorbed on the surface of a **stationary solid phase**.

Accordingly, it is of two types:

1. **Liquid–solid chromatography:** When a liquid is used as mobile phase, e.g. TLC and HPLC.
2. **Gas–solid chromatography:** If the mobile phase is gas, e.g. gas chromatography.

   Equilibration between the mobile and the stationary phase accounts for the separation of different solutes.

   - It involves analytical separation of a chemical mixture (gas or liquid) by passing it over an adsorbent bed that adsorbs different compounds at different rates.
   - Solute in liquid or gas phase interacts with adsorption sites on solid surface, finely divided particles, for maximum surface area.

Stationary Phase

The ideal adsorbent must fulfil the following requirements:

- Insoluble in mobile phase.
- Inert to solutes (adsorptive).

- Colourless, especially when working with coloured mixtures.
- Suitable particle size enough to give good separation and reasonable flow rate.

Some examples of adsorbents are:

Silica gel

It is the most widely used adsorbent in both column and thin layer chromatography. Silica gel is prepared by acidification of sodium silicate with sulphuric acid, followed by washing with water, and drying.

Alumina

It is aluminium oxide (Al_2O_3), alumina activated by heating at 400°C, overnight. It has large capacity, insoluble, relatively inert and easy availability.

Charcoal

There are two types of charcoal, based on temperature of activation called **non-polar** (prepared by activation at 1000°C) and **polar charcoal** prepared at lower temperature and contains water.

Kieselguhr (Diatomaceous earth)

It has relatively low adsorption power.

COLUMN CHROMATOGRAPHY

Column or classical liquid chromatography is extensively useful in quantitative analysis and in sample purification. Glass columns (the dimensions are dictated by the amount of the sample) filled with the stationary phase (adsorbent, ion exchange or affinity media) are commonly used. The sample is applied as a narrow zone at the top of the column and the mobile phase is passed through the column, normally, under atmospheric pressure. The eluted sample is collected in fractions of small volumes, for analysis.

ION EXCHANGE CHROMATOGRAPHY

Ion exchange chromatography or **ion chromatography** is a process that separates ions and polar molecules, based on their affinity to the ion exchanger. It works on, almost, any kind of charged molecule including large proteins, small nucleotides and amino acids. It is often used in protein purification, water analysis and quality control. The water soluble and charged molecules, such as proteins, amino acids and peptides, bind to moieties, which are oppositely charged, by forming ionic bonds to the insoluble stationary phase. The equilibrated stationary phase consists of an ionizable functional group, where targeted molecules of a mixture, to be separated and quantified, can bind while passing through the column (Fig. 37.1).

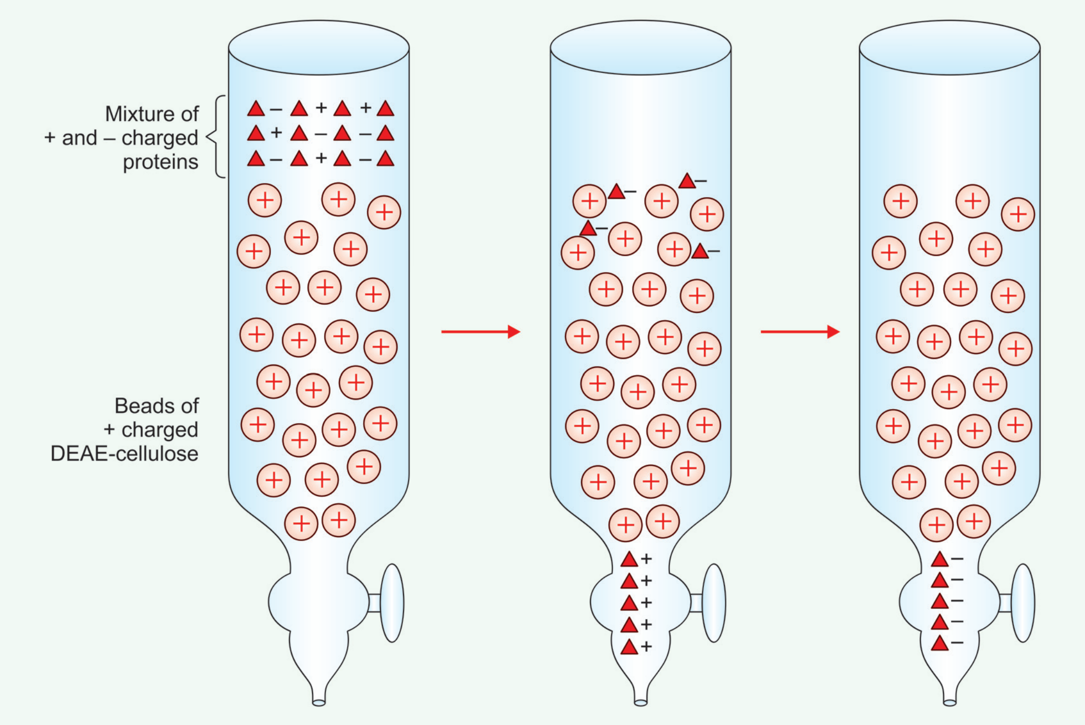


Fig. 37.1: Ion exchange chromatography

This method applies the idea of the interaction between molecules and the stationary phase, which are charged oppositely to each other.

- Ion exchange resins may be defined as polyvalent material that is insoluble in an aqueous medium and contains functional groups that are capable of exchanging ions from the surrounding medium. Synthetic resins, based on a polystyrene matrix, are most commonly employed. The principle of separation depends on the differential affinity of the charged particles, for the functional group of ion exchange resins.
- **Cation exchange chromatography** is used when the desired molecules, to separate, are cations while **anion exchange chromatography** is used to separate anions, meaning that the beads in the column contain positively charged functional groups to attract the anions. The bound molecules then can be eluted and collected using an eluent, which contains anions and cations by running higher concentration of ions through the column or changing pH of the column.
- One of the primary advantages for the use of ion chromatography is only one interaction involved during the separation, as opposed to other separation techniques. Therefore, ion chromatography may have higher matrix tolerance. However, there are also disadvantages involved when performing ion-exchange chromatography, such as constant elution with the technique which leads to the inconsistency from column to column.

Clinical Utility

- Ion exchange resins (IER) have been widely used, especially in medicines, due to its high capacity and the uncomplicated system of the separation process. Ion exchange chromatography plays a central role in the investigation of amino acid composition of proteins and the determination of free amino acids in physiological fluids. The amino acid mixture is passed through a column, packed with the resin, and the amino acids are eluted by buffers of increasing pH through the column. Amino acids move down the column as separate bands. The effluent containing individual amino acids is then allowed to react with ninhydrin reagent, to form a colored-complex. The intensity of this colored-complex is continuously monitored as it passes through the flow cell of an absorptiometer linked to an electronic recorder. The amino acids are detected as peaks with areas proportional to the respective amounts present in the mixture.
- It is also used for the measurement of HbA1c, porphyrin and in water purification.
- One of the clinical uses is to use ion exchange resins for kidney dialysis. This method is used to separate blood elements by using the cellulose membrane-artificial kidney.
- Another clinical application of ion chromatography is in the rapid anion exchange chromatography technique used to separate creatine kinase (CK) isoenzymes from human serum and tissue supplied on autopsy.

GEL FILTRATION CHROMATOGRAPHY

One of the most useful and powerful tool of separating proteins, from each other, on the basis of size, is **molecular exclusion chromatography**. It is also known as **gel filtration** or **molecular sieve chromatography**.

It differs from ion exchange chromatography which separates solutes on the basis of their electric charges and acid base properties. Common column materials are Sephadex, Agarose (the commercial name of a polysaccharide derivative), and Bio-gel (a commercial polyacrylamide derivative), all of which can be prepared with different degrees, and thus, travel down the column at different rate. Very large protein molecules cannot enter the pore of the bead. They are excluded and, thus, remain in the exclusion volume of the column, defined as the volume of the aqueous phase outside the beads. On the other hand, small proteins are retarded by the column while large proteins pass through rapidly. Proteins of intermediate size will be excluded from the beads to a degree that depends on their size, hence, the term exclusion chromatography. An elution curve can be constructed from measurements of the protein concentration in small fractions of elute.

In molecular exclusion chromatography, the mixture of proteins, dissolved in a suitable buffer, is allowed to flow by gravity, down a column packed with beads of an inert, highly hydrated, polymeric material that has previously been washed and equilibrated with the buffer (Fig. 37.2).

It can also be used to separate macromolecules as well as very large bio-structures, e.g. ribosomes, cell nuclei and bacteria, by using gels with different degrees of internal porosity.

The resolving power of molecular exclusion chromatography is now widely used for determining the molecular weight of proteins.

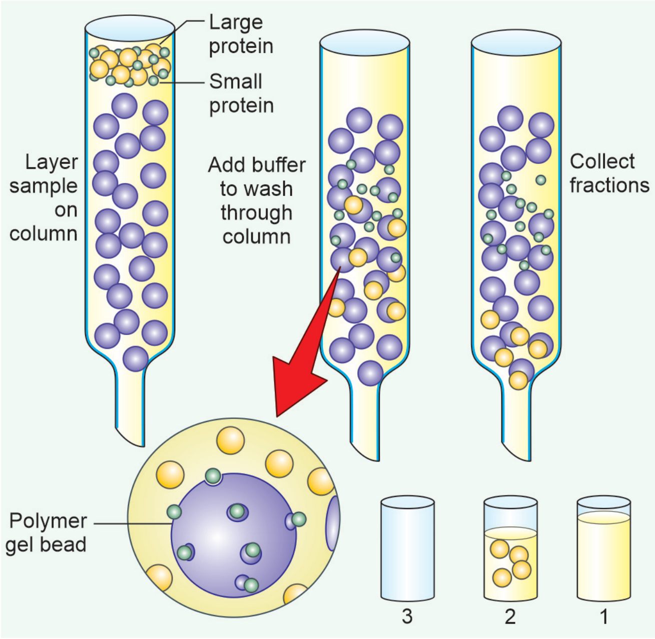


Fig. 37.2: Gel filtration chromatography

AFFINITY CHROMATOGRAPHY

Affinity chromatography exploits the ability of enzymes and some other proteins to bind substrates, coenzymes, inhibitors, activators and other ligands, specifically and reversibly. The concept is realized by covalently binding of the ligand to an insoluble matrix and packing the support into a chromatographic column. In principle, only enzymes or proteins, which display appreciable affinity for the immobilized ligand will be retained on such a column, while others displaying little or no affinity will pass through, unretarded. The specifically adsorbed protein can subsequently be released by altering the mobile phase to favor dissociation (Fig. 37.3).

PARTITION CHROMATOGRAPHY

Partition chromatography is a chromatographic separation process whereby compounds are partitioned between two liquid phases, similar to the separatory funnel, liquid-liquid separation dynamic. In liquid-liquid separation, a compound is distributed between two immiscible liquid phases under equilibrium conditions. It is one of the most useful biochemical laboratory techniques used to separate different bio-molecules, like proteins, carbohydrates, lipids, etc. It is based on differences in retention factor as well as distribution coefficient of the analytes using liquid for both stationary as well as the mobile phase.

Partition chromatography can be divided into liquid–liquid chromatography and bonded-phase liquid chromatography.

- **Liquid–liquid chromatography** uses liquid and the stationary phase is attached to a supporting matrix by physical means, i.e. no chemical bond. For example, cellulose, starch or silica matrix is used to support water stationary phase and all these have the ability to bind physically as much as 50% water (w/v) and remain free floating powder. There are some advantages of liquid–liquid chromatography as, e.g. silica, cellulose or starch, used are cheap and have a high capacity with broad selectivity.

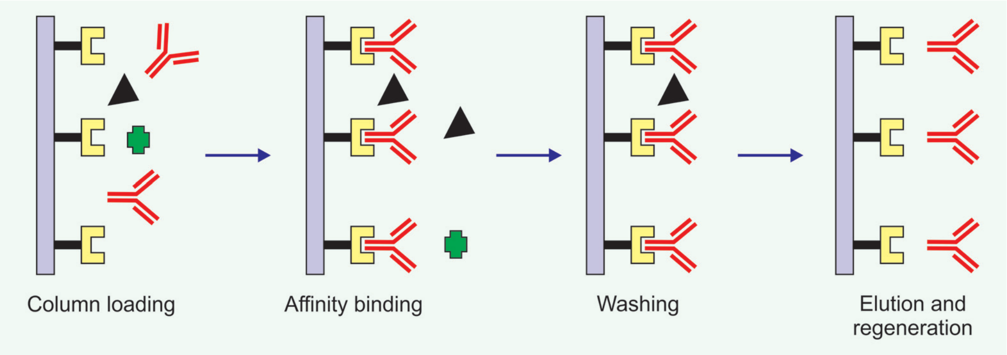


Fig. 37.3: Affinity chromatography

However, there are some disadvantages too, like elution of the sample may gradually remove the stationary phase, thereby altering the chromatographic condition.

- To overcome these problems bonded phases are used, like in **bonded-phase liquid chromatography.** Silica is derivatized to the immobilized stationary phase by using reaction with an organochlorosilane. Thus, stationary phase will not change with elution.
- Normal phase liquid chromatography uses **polar stationary phase** and relatively **non-polar mobile phase**. Alkylamine bonded to silica is most commonly used as a stationary phase while organic solvents like hexane, heptane, dichloromethane or ethylacetate are used as mobile phase. These solvents are used in such a way to form an elutropic series based on their polarity, with increasing polarity. The order of elution of analytes is performed in such a way that the least polar analyte is eluted first and the most polar analyte is eluted at the end.
- Column or classical liquid chromatography is extensively useful in quantitative analysis and in sample purification. Glass columns (the dimensions are dictated by the amount of the sample) filled with the stationary phase (adsorbent, ion exchange or affinity media) are commonly used. The sample is applied as a narrow zone at the top of the column and the mobile phase is passed through the column normally under atmospheric pressure. The eluted sample is collected in fractions of small volumes for analysis.

PAPER AND THIN LAYER CHROMATOGRAPHY

Paper chromatography and thin layer chromatography (TLC) techniques are easiest to perform and require simple apparatus. They readily provide qualitative information. However, with careful attention, it is possible to obtain quantitative data. In the simplest form of the technique, a narrow zone or spot of the sample mixture, to be separated, is placed near one end of the paper strip or TLC plate and allowed to dry. The strip or plate is then placed with this end dipping in the solvent mixture, taking care that the sample spot (zone) is not immersed in the solvent. As the solvent moves towards the other end of the strip, the test mixture separates into various spots. This is known as **development**. The strip or plate is removed after an optimal development time and dried, and the spots/zones are detected using a suitable reagent. The paper or silica gel acts as an inert support, the interstices of which hold the more polar phase (usually aqueous) of the solvent mixture that, thus, acts as the ***stationary phase*** while the less polar phase acting as the ***mobile phase***. Separation results from differences in partition equilibrium of the components in the mixture. However, the paper or silica gel interacts (adsorption and some degree of ion exchange) with the components and effects the separation.

An important characteristic, used in paper and thin layer chromatography, for the identification of the compounds, is called the **Rf value.**

$$\text{Rf} = \frac{\text{Distance moved by the substance from the origin}}{\text{Distance moved by the solvent-front from the origin}}$$

Paper and thin layer chromatography techniques are immensely versatile and one can readily alter the various parameters to achieve a particular separation. These techniques have, therefore, become indispensable in biomedical research. There are different ways in which chromatograms are run. They are one-dimensional (ascending or descending), circular or horizontal and two-dimensional.

Ascending Chromatography

After the material has been spotted on a chromatographic paper (usually 38 × 38 cm), the paper is folded into a cylindrical form and stitched at the joint, at three places. The paper cylinder is saturated with the solvent vapor for 1 to 2 h before dipping into the solvent system, which is kept in a Petri dish and enclosed by a bell jar. The unit is kept on a clean glass plate and sealed with modeling clay, all around, to make it airtight. Usually, the solvent is allowed to run overnight. The next day, the paper is taken out, the solvent front is marked with a lead pencil and air dried to remove last traces of the solvent.

Descending Chromatography

This requires the solvent to be kept at the top, in a trough and the end of the paper, close to the spotting line, is dipped into the solvent. The solvent flows downward.

Two-dimensional Chromatography

A margin of 3.8–5.0 cm is made on both sides and the material under test is spotted at the junction of these margins. The paper is irrigated in the first solvent for 18 h, air dried and then run at right angle to the first run in the second solvent. The drawback of this technique is that only one sample can be separated in each run while the advantage is that it has good resolution.

GAS–LIQUID CHROMATOGRAPHY

Gas chromatography, also called **gas–liquid chromatography** (**GLC**), is the method of choice for rapidly and accurately analyzing volatile substances.

- In essence, the volatile material is injected into a column, containing a liquid adsorbent (**stationary phase**), supported on an inert solid.
- The basis for the separation of the component of volatile mixture is the difference in the partition coefficient of the components, as they are carried through the column by an inert gas (**mobile phase**), such as nitrogen, helium or argon etc.
- The resolved compounds are carried over to the detector, which presents an electrical signal to the recorder that, in turn, displays the separated components of the mixture as a series of peaks. These peaks are detector responses versus time. The area under these peaks is proportional to the amount of substance passing through the detector.
- Retention time is the time interval measured from the time of injection of the sample to the time when peak appears. Unknown samples are identified based on the retention time of known standard samples.
- GLC has been extensively used for the estimation of fatty acids, bile acids, steroids, hormones, sugars and alkaloids.
- Gas chromatography is in principle similar to column chromatography (as well as other forms of chromatography, such as HPLC and TLC) but has **several notable differences**.
  - First, the process of separating the compounds in a mixture is carried out between a liquid stationary phase and a gas mobile phase, whereas in column chromatography the stationary phase is a solid and the mobile phase is a liquid. Hence the full name of the procedure is gas–liquid chromatography, referring to the mobile and stationary phases, respectively.
  - Second, the column through which the gas phase passes is located in an oven where the temperature of the gas can be controlled, whereas column chromatography (typically) has no such temperature control.
  - Finally, the concentration of a compound in the gas phase is solely a function of the vapour pressure of the gas.
- Gas chromatography is, however, similar to fractional distillation, since both processes separate the components of a mixture, primarily, based on boiling point (or vapor pressure) differences. However, fractional distillation is typically used to separate components of a mixture on a large scale, whereas GC can be used on a much smaller scale.

HIGH PRESSURE LIQUID CHROMATOGRAPHY (HPLC)

High pressure liquid chromatography, also referred to as high performance liquid chromatography (HPLC) is one of the major methods of analysis of organic compounds. It offers a high degree of analytical selectivity in addition to the great speed of separation. Its versatility and sensitivity are equaled only by gas–liquid chromatography but it has an edge over the latter in that its scope is not limited by sample volatility or thermal stability.

The technique of HPLC involves the separation of the various constituents in a sample followed by their individual detection and quantitation. All chromatographic modes (adsorption, partition, ion exchange and molecular sieving) are possible. High separation efficiency is achieved by optimization of column parameters, particularly, the particle size of the column packing. Particles of size of 5–10 μm are commonly employed (against the 100 μm particles used in classical liquid chromatography). This has necessitated the use of high pressure to obtain adequate flow rate of mobile phase. Detection system forms part of HPLC. Ultraviolet, fluorescence and refractive index detectors are most commonly employed for the purpose.

Primary Components of HPLC Instrument

1. **Solvent reservoir:** Mobile phase solvents are contained in a glass reservoir.
2. **Pump:** The pump aspirates the mobile phase (containing the sample) from the solvent reservoir and forces it through the column and detector.
3. **Sample injector:** An aliquot of sample is introduced into a liquid chromatograph through the sample injector.
4. **Column:** Silica, alumina, charcoal and organic polymers are used as stationary supports, which are packed in stainless steel or glass-lined stainless steel tubes.
5. **Detector:** UV light, visible light and diode array absorbance detectors are used in HPLC system. Fluorometers and electrochemical detectors are also used. UV and visible photometers absorb radiant energy emitted by compounds as they elute from chromatographic column.

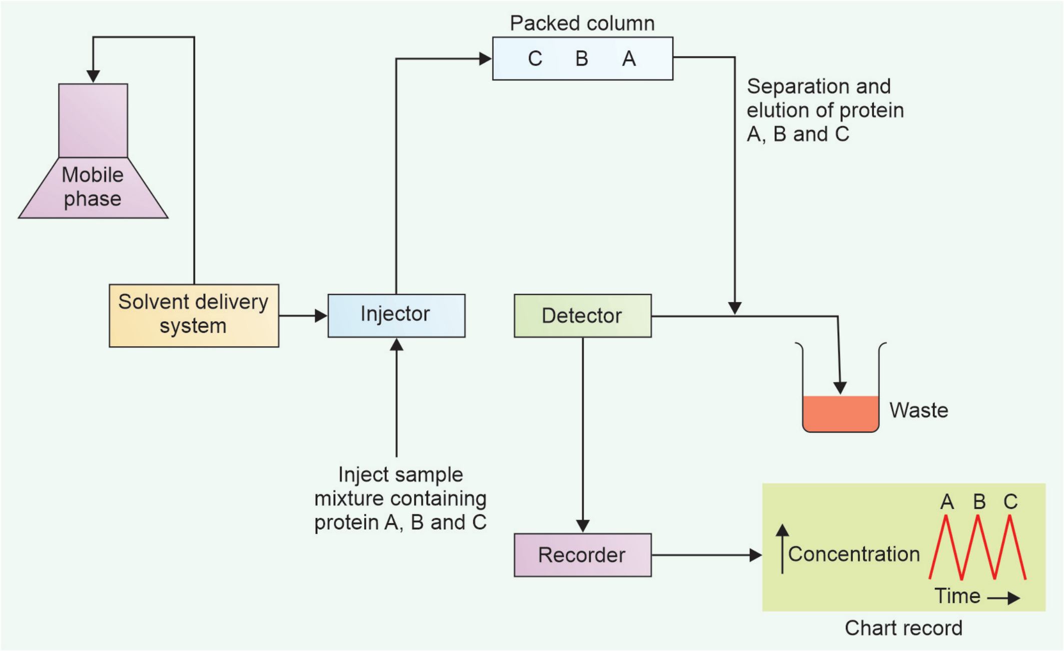

Packed column
C B A
Separation and elution of protein A, B and C
Mobile phase
Solvent delivery system
Injector
Detector
Waste
Inject sample mixture containing protein A, B and C
Recorder
A B C
Concentration
Time
Chart record

Fig. 37.4: Primary components of HPLC instrument

6. **Recording device**.
7. **Microprocessor**.

The area of each chromatographic peak is determined from stored data and is used to compute the concentration of the solute (Fig. 37.4).

Advantages

Highly specific, precise and accurate. It is a common technique used in therapeutic drug monitoring.

Disadvantages

Quite expensive technique.

SEPARATION OF SUGARS BY PAPER CHROMATOGRAPHY

- Take a strip of Whatman No.1 filter paper, about 50 cm long. The width should be such that it can fit the tank. Mark the required number of spots. Draw a pencil line atleast 10 cm from one end and parallel to it. Place the spots 2 cm apart with standards on each side of a test of the same specimen, to allow staining with more than one stain. Apply 5 to 10 mL of the standard solution. Allow to run for 16–17 hours.
- Remove from the tank, blot between filter papers and blow-off the remaining solvent in a stream of cold air. The paper is then ready for staining, which can be done either by dipping in a bath or spraying.
- The aniline-diphenylamine reagent is a useful one. Dip the strip in this, heat carefully for a few minutes at 95–100°C. The spots show useful differences in colour (pentoses being grey brown, fructose, sucrose and glucuronic acid brown, glucose and galactose a greenish-blue, lactose and maltose are blue).
- With the silver nitrate stain, dip the paper into the silver nitrate solution for a minute or two, dry in air and then dip in the ethanolic sodium hydroxide. Brown spots appear almost immediately and intensify over the next few minutes (Fig. 37.5).

PRACTICAL EXERCISE

1. Separation of amino acids by paper chromatography.

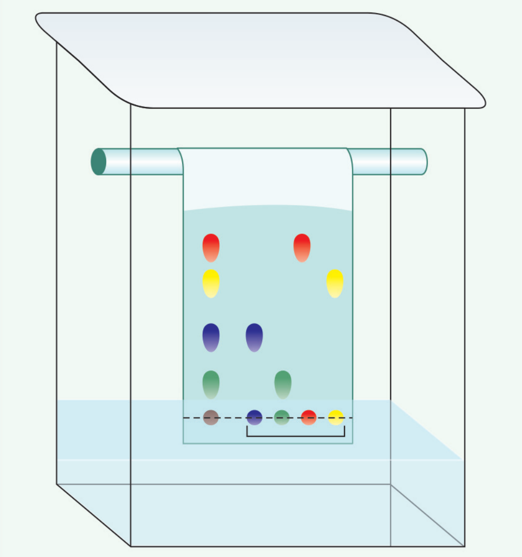


Fig. 37.5: Paper chromatography

Important Questions

1. Define chromatography. Describe various types of chromatography.
2. Write notes on:
   i. Ion exchange chromatography
   ii. Thin layer chromatography
   iii. HPLC

Chapter

38

Electrophoresis

The term electrophoresis means, Electro = electric field + Phoresis = migration. So, as the name indicates, electrophoresis is a method of separation where charged molecules migrate in differential speeds in an applied electric field.

- The charged molecules under the influence of electric field migrate towards oppositely charged electrodes. Those molecules with positive (+ve) charge move towards cathode while negative (–ve) molecules move towards anode.
- The migration is due to charge on the molecules and potential applied across the electrodes.
- The sample, under test, is placed at one end, near one of electrodes. When current is applied, the molecules start moving to respective electrodes.
- The movement is influenced by **molecular weight** of the molecule. So, when a mixture is placed on the electrophoresis paper or agarose gel, different bands are seen along the paper after the process. This is due to differential rate of movement by molecules, based on their weight. Those with high molecular weight move slower while those with small weight move faster.
- The **size of the molecule** also influences the movement. The bigger size molecule experiences more friction than smaller ones in motion. These molecules migrate at different speed and to different length, based on their charge, mass and shape.
- Electrophoresis is one of the widely used techniques in molecular biochemistry, microbiology and biomedical research. It is one of the highly efficient techniques of analysis and sole method for separation of proteins for western blot and RNA studies.

ELECTROPHORESIS SYSTEMS

There are two types of gel electrophoresis systems, referred to as **horizontal electrophoresis system** and **vertical electrophoresis system**. Each system requires the equipment, which consists, basically, of two items, i.e. a **power pack** and an **electrophoresis unit**.

Power Pack

Power pack provides a stabilized direct current and controls both voltage (0–500 V) and current output (0–150 A).

The power pack supplies a direct current between the electrodes in the electrophoresis unit. Electrophoresis is carried out in an appropriate buffer, which is essential to maintain a constant state of ionization of the molecules being separated. Any variation in pH will alter the overall charge and hence, the mobilities (rate of migration in the applied field) of the molecules being separated.

Electrophoresis Unit

Electrophoresis unit consists of electrodes, buffer reservoir, supporting medium and transparent insulating cover. Electrodes are made of platinum or stainless steel.

Types of electrophoresis units

Electrophoresis units are available for running either **vertical** or **horizontal** gel systems.

Horizontal type

It consists of two buffer reservoirs (compartments), which are placed side by side to each other. Separate compartments are necessary because any change in pH, occurring at electrodes, does not affect the buffer that is in contact with the supporting medium (Fig. 38.1).

- It has a transparent insulating cover which provides electrical insulation and also prevents evaporation of buffer. The supporting medium is cast on a glass/plastic sheet and is placed on a cooling plate.
- Support for electrophoresis medium (supporting medium) may be paper, cellulose acetate or gel.
- Contact between the supporting medium and the buffer reservoir is through a wick.

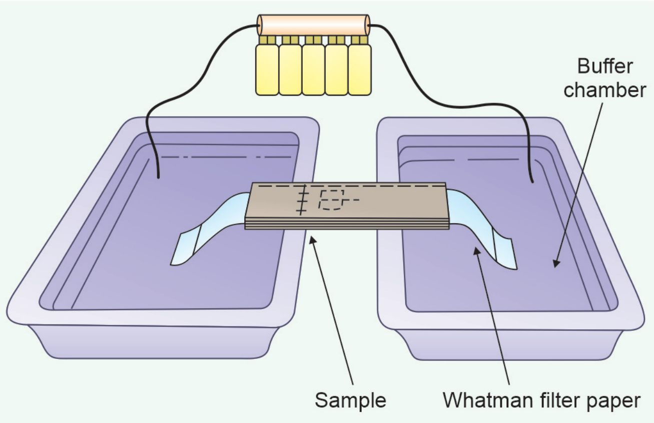


Fig. 38.1: Horizontal electrophoresis unit

Vertical Type

Vertical slab gel units are commercially available and routinely used to separate proteins in acrylamide gel. Electrophoresis unit, required for vertical system, however, differs from the horizontal type:

- There are two buffer reservoirs where one is placed above the other and may be referred to as the upper buffer reservoir and the lower buffer reservoir.
- The supporting medium is gel, especially polyacryl amide gel, which is formed between the two glass plates that are clamped together but held apart by plastic spacers.
- The plastic comb (well-forming template) is placed in the gel solution and is removed after polymerization, to provide loading wells for samples.
- Gel dimensions are usually 12 × 14 cm, with a thickness of 1–2 mm.
- Transparent insulating chamber provides an electrical insulation, i.e. when the apparatus is assembled, the electrophoresis tank buffer surrounds the gel plates and affords some cooling to the gel plates.
- During electrophoresis, the upper part of the gel is in contact with upper buffer reservoir and lower part of the gel is in contact with the lower buffer reservoir.

TYPES OF ELECTROPHORESIS

Electrophoresis can be broadly divided into 2 types, designated as slab electrophoresis and capillary electrophoresis.

SLAB ELECTROPHORESIS

It is the classical method, which is widely used for industrial use. It is slow, time consuming and bulky. Yet, it is the sole method available for separation of proteins, enzymes, hormones, antibodies and nucleotides.

Based on the principle used for separation, it is further divided into 3 types, as zone electrophoresis, isoelectric-focusing and immuno-electrophoresis.

ZONE ELECTROPHORESIS

Here the charged particles are separated into different zones or bands. This is of two types, referred to as paper electrophoresis and gel electrophoresis.

Paper Electrophoresis

It employs a Whatman filter paper No. 1, which is moistened by a buffer and then connected at two ends, to the oppositely charged electrodes.

- The sample is applied on one end and let for separation of the components under electric gradient.
- After separation, paper is dried and stained to get colored bands, which are later recognized by comparing with the standard.
- For a sample of normal serum, 5 bands of proteins are visualized by paper electrophoresis (Fig. 38.2).

Cellulose Acetate Electrophoresis

Cellulose acetate has the advantage over paper in the way that it is a much more homogeneous medium, with a uniform pore size and does not absorb proteins in the way that paper does. Thus, resolution is better.

- Single sample may be run on cellulose acetate strips of 2.5 cm × 12 cm size, whereas multiple samples can be run on wider sheets.
- The cellulose acetate is allowed to wet in buffer (pH 8.6 for serum), and thereafter, the sample (1–2 mm^3) is loaded.

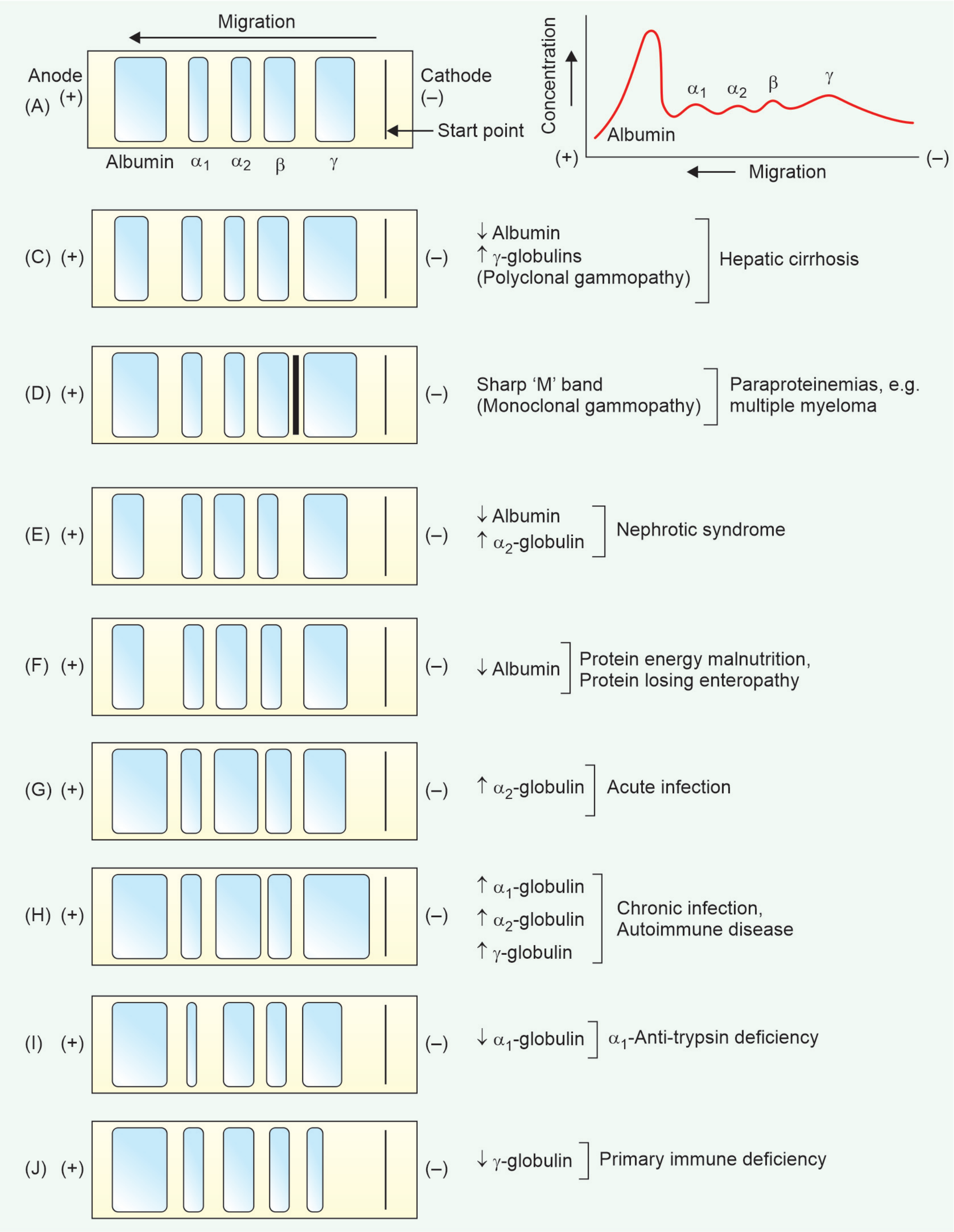

Migration
Anode
(A) (+)
Cathode
(–)
Start point
Albumin α_1 α_2 β γ
Concentration
(+)
(–)
Migration
Albumin
α_1 α_2 β γ
(C) (+) (–)
↓ Albumin
↑ γ-globulins
(Polyclonal gammopathy)
Hepatic cirrhosis
(D) (+) (–)
Sharp 'M' band
(Monoclonal gammopathy)
Paraproteinemias, e.g. multiple myeloma
(E) (+) (–)
↓ Albumin
↑ α_2-globulin
Nephrotic syndrome
(F) (+) (–)
↓ Albumin
Protein energy malnutrition, Protein losing enteropathy
(G) (+) (–)
↑ α_2-globulin
Acute infection
(H) (+) (–)
↑ α_1-globulin
↑ α_2-globulin
↑ γ-globulin
Chronic infection, Autoimmune disease
(I) (+) (–)
↓ α_1-globulin
α_1-Anti-trypsin deficiency
(J) (+) (–)
↓ γ-globulin
Primary immune deficiency

Fig. 38.2: Pattern of bands of serum proteins after separation by paper electrophoresis

- The ends of the strip make contact with the buffer tanks, via a filter paper wick that overlaps the ends of the cellulose acetate strip.
- The electrophoresis is conducted at 6–8 V cm^{-1}, for about 3 h.
- Thereafter, the strip is stained for proteins, destained and the bands are visualised.

Gel Electrophoresis

It is similar to paper electrophoresis, wherein instead of paper, a gel, made of agarose or SDS (sodium dodecyl sulphate), is used. It is a method for separation and analysis of macromolecules (DNA, RNA and proteins) and their fragments, based on their size and charge.

In gel electrophoresis there are two factors which are involved in separation. These are:

1. The application of an electric current, which produces separation on the basis of **charge density** (rate of migration in the electric field).
2. **Molecular sieving** (separation based on pore size of the material through which the components of a mixture migrate). Thus, an increase in concentration of the solid phase in the gel will result in a decrease in the pore size and retention of large molecules.

Electrophoresis Gel

The term gel refers to the matrix used to contain and then separate the target molecules. In most cases, the gel is a cross-linked polymer, whose composition and porosity is chosen based on the specific weight and composition of the target to be analyzed.

TYPES OF GEL

Most commonly used gels are of three types, i.e. **starch, agarose** and **polyacrylamide gels**. Each type of gel is well-suited to different types and sizes of analytes.

Starch Gel Electrophoresis

Starch gel is one of a wide variety of supporting media that is used for horizontal zone electrophoresis. Such gels are prepared by heating and cooling, partially hydrolyzed potato starch in an appropriate buffer solution. The choice of buffer is empirical and a wide variety of compositions can be used.

- Starch gels are slightly more opaque than acrylamide or agarose. Nondenatured proteins can be separated according to charge and size. They are visualised using Amido Black staining. Typical starch gel concentration used is 5% to 10%.
- Homemade glass plates provided with thin-glued edges, 3 mm high, are generally used for this purpose. Plates of 16 × 15 cm are suitable for an average of 10–15 samples per run.
- Gel is carefully poured to the glass tray, from the centre to the corners and allowed to solidify for 30–40 minutes, before loading the samples.
- Both the buffer tanks are filled with buffer.
- A glass cover is placed and the power supply is connected with the electrodes of the buffer tanks.
- The sample migrates from –ve to +ve pole and runs for 4–6 hours, at 0.3 kV and 7 mA.
- Switch off the power supply and the cooling system.
- Remove the loading papers from the gel, using tweezers and place the cover glass back on the gel.
- Separate the upper part of the gel from the main body by cutting the gel with a steel wire leaning on the edges of the plate and remove the upper part of the gel. Free the gel from the edges of the plate using the tip of a Pasteur pipette.
- Place the plate in a staining tray and add the staining solution. Remove the staining solution after about 20 minutes. Rinse once with 200 mL of 50% methanol and dry the starch surface with tissue paper.

Agarose Gel Electrophoresis

Agarose is one of the components of agar that is a mixture of polysaccharides isolated from certain seaweeds. It is a linear polysaccharide (average relative molecular mass about 12 kD) made up the basic repeat unit agarobiose, which comprises alternating units of the galactose and 3,6-anhydrogalactose. Agarose is usually used at a concentration between 1% and 3%.

- Agarose gels are formed by suspending dry agarose in aqueous buffer, then boiling the mixture until a clear solution is formed. This is poured and allowed to cool to room temperature to form a rigid gel. The pore size in the gel is controlled by the initial concentration of agarose; large pore sizes are formed from low concentration and smaller pore sizes are formed from high concentrations.
- Agarose gels are used for the electrophoresis of, both proteins and nucleic acids. For proteins, the pore sizes of a 1% agarose gel are large relative to the size of proteins.
- Agarose gels are used in techniques such as immuno-electrophoresis or flat-bed isoelectric focusing, where the proteins are required to move unhindered in the gel matrix according to their native charge.

Polyacrylamide Gel Electrophoresis (PAGE)

Polyacrylamide gels consist of long chain molecules of acryl-amide with varying proportion of cross-linkages resulting from the addition of a proportion of bis-acrylamide before polymerization. The porosity of the gel is inversely proportional to the extent of cross-linking between the polymers of acrylamide. Polymerization of acrylamide occurs rapidly at room temperature in the presence of ammonium persulphate and tetramethyl-ethylene-diamine (TEMED).

- Polyacrylamide gel electrophoresis is used for separating proteins ranging in size from 5 to 2,000 kDa, due to the uniform pore size provided by the polyacrylamide gel. Pore size is controlled by modulating the concentrations of acrylamide and bis-acrylamide powder, used in making the gel. Care must be taken when creating this type of gel, as acrylamide is a potent neurotoxin.

- Traditional DNA sequencing techniques use polyacrylamide gels to separate DNA fragments differing by a single base-pair in length so the sequence could be read. It is currently most often used in the field of immunology and protein analysis, often to separate different proteins or isoforms of the same protein into separate bands. These can also be transferred to a nitrocellulose membrane, to be probed with antibodies and corresponding markers, such as in a western blot.

Sodium Dodecyl Sulphate-polyacrylamide Gel Electrophoresis (SDS-PAGE)

SDS-polyacrylamide gel electrophoresis (SDS-PAGE) is the most widely used method for analyzing protein mixtures, qualitatively. It is, particularly, useful for monitoring protein purification and, as the method is based on the separation of proteins according to size, to determine the relative molecular mass of proteins.

Isoelectric-focusing

Here, the isoelectric pH is set at different foci and hence, the molecules are immobilized to their isoelectric point. They do not move towards electrodes but stay at a specific isoelectric pH. This is more efficient to separate proteins.

Immuno-electrophoresis

This is the combination of electrophoresis with immune reaction. First, proteins are separated on to the electrophoresis paper, then the antibodies are allowed to diffuse through the paper and react with separated protein molecules in bands.

SEPARATION OF SERUM PROTEINS BY PAPER ELECTROPHORESIS

Principle

In paper electrophoresis, a strip of filter paper is dipped in a buffered solution (barbitone buffer, pH 8.6) and is laid on a platform. Two ends of the strip are dipped in the buffer. Entire apparatus is covered with a sheet, to minimize evaporation. Two electrodes are also dipped in two compartments containing buffer. Current is supplied through a power supply, which either supplies constant current and/or voltage. Current is carried by the charged particles and depending upon the charge, protein migrates towards either anode or cathode.

Procedure

About 10 μL of serum is applied along a thin line, near the cathode. Since at pH 8.6, all the proteins carry a net negative charge and tend to migrate towards the anode, thus, on passing a constant current (about 1–2 mA per strip), different proteins migrate at different rates. At the end, their positions are revealed by some dye, such as bromophenol blue, which stains proteins. Different proteins form separate bands on the paper strip.

Interpretation

In the normal serum five bands are observed. Since albumin carries largest number of the negative charges, it moves fastest while α-globulin has less negative charges and moves the least. Starting from anode, these bands are designated as albumin, α_1-globulin, α_2-globulin, β-globulin and γ-globulin.

Pattern of serum protein on electrophoresis, may be used in the diagnosis of a disease, e.g. multiple myeloma (Fig. 38.2).

Agarose Gel Electrophoresis

Preparation of the Gel and Slides

To 0.3 g of agarose, add 30 mL of barbitone buffer (0.2 M, pH 8.6, containing 0.9% sodium azide). Heat in a boiling water bath for 15 to 20 min with shaking. Place 8 microscopic slides on a levelled sheet and put 3 mL of agar on each slide. Allow to set at room temperature for 15 min and store in a moist air-tight chamber. Punch holes in the gel.

Apply 5 mL of serum carefully inside the well and add 1 μL of the dye bromophenol blue. The outer compartments of the electrophoresis chamber are filled with the buffer. Place the slides on the centre support and connect to the buffer compartments with Whatman 3 filter paper strips. Cover the chamber, apply current of 10–12 volts/cm and leave for 3 hours. Disconnect the electrodes and remove the slides. Wash the slides 3 times with distilled water (5 min/wash) and dry overnight at 37°C. Stain for 5–10 min in amido black 10B in solvent (methanol: water: acetic acid – 4 : 4 : 1) and destain with 10% aqueous acetic acid.

Polyacrylamide Gel Electrophoresis

Polyacrylamide gels consist of long chain molecules of acryl-amide with varying proportion of cross-linkages resulting from the addition of a proportion of bisacrylamide before polymerization. The porosity of the gel is inversely proportional to the extent of cross-linking between the polymers of acrylamide.

Formation of polyacrylamide gel by polymerization of monomer acrylamide and bisacrylamide occurs only in the presence of free radicals. Polymerization of acrylamide occurs rapidly at room temperature in presence of ammonium persulphate and tetramethylethylenediamine (TEMED).

Reagents

1. Acrylamide:bisacrylamide (30 : 0.9 g/100 mL)
2. Tris-HCl buffer (1.5 M, pH 8.8)
3. TEMED
4. Electrophoresis buffer: Tris-glycine, pH 8.6

Mix 10.2 mL of acrylamide: bisacrylamide, 8.7 mL of tris-HCl buffer, 16 mL of water and 0.02 mL of TEMED, just before the experiment. Mix quickly and add 0.17 mL of freshly prepared 10% ammonium persulphate with a Pasteur pipette. Make sure that there are no air bubbles. Apply 5 µL of serum, diluted with 45 µL of sucrose and add 1 µL of bromophenol blue as indicator.

PRACTICAL EXERCISE

1. Perform electrophoresis for serum proteins and interpret the bands.

Important Questions

1. Define electrophoresis. Describe various types of electrophoresis.
2. Write notes on:
    i. Gel electrophoresis
    ii. PAGE
    iii. Isoelectric focusing

Section 3

Clinical Biochemistry

39. Enzyme Linked Immunosorbent Assay
40. Quality Control
41. Automation in Clinical Chemistry Laboratory
42. Organization of a Clinical Chemistry Laboratory
43. Pancreatic Function Tests
44. Liver Function Tests
45. Gastric Function Tests
46. Kidney Function Tests
47. Thyroid Function Tests
48. Quantitative Estimation of Some Biochemical Constituents in Urine
49. Minerals
50. Hormone Profiles
51. Special Biochemical Investigations
52. Biochemical Analysis of CSF
53. Biochemical Analysis of Ascitic and Pleural Fluids
54. Biochemical Examination of Stool

Chapter

39

Enzyme Linked Immunosorbent Assay

Enzyme linked immunosorbent assay (**ELISA**) is an antibody test, a test for immune response to immunogen which attack the body, such as virus, bacteria and allergens that may be related to certain infectious conditions, e.g. to diagnose HIV which causes AIDS.

Applications of ELISA

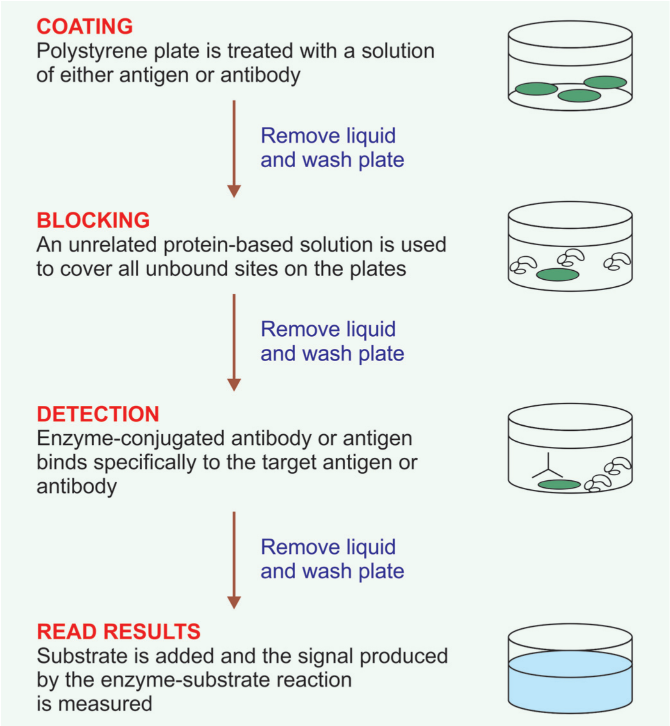
ELISA tests are used as *in vitro* diagnostics in medical laboratories, such as for the:

- Estimation of hormones and tumor markers
- Detection of *Mycobacterium* antibodies in tuberculosis, hepatitis B markers and HIV antibodies in blood samples
- Detection of rotavirus in faeces, etc.
- Besides, these are widely used in research, drug discovery, bioassay validation, quality control and manufacturing processes in the pharmaceutical and biotechnological industry and academic organizations.

General Principle of ELISA

ELISA is a very sensitive immunochemical technique which is used to access the presence of specific protein (antigen or antibody) in the given sample. It requires at least one antibody with specificity for the particular antigen. An enzyme conjugated with an antibody reacts with a colorless substrate to generate a colored reaction product (Fig. 39.1).

Some of the commonly used enzymes and their substrates are listed in Table 39.1.


COATING
Polystyrene plate is treated with a solution of either antigen or antibody

Remove liquid and wash plate

BLOCKING
An unrelated protein-based solution is used to cover all unbound sites on the plates

Remove liquid and wash plate

DETECTION
Enzyme-conjugated antibody or antigen binds specifically to the target antigen or antibody

Remove liquid and wash plate

READ RESULTS
Substrate is added and the signal produced by the enzyme-substrate reaction is measured

Fig. 39.1: General principle of ELISA

Table 39.1 Commonly used enzymes and their substrates

Enzyme	Substrate	Chromogen
Horseradish-peroxidase (HRP)	*o*-Phenylenediamine-dihydrochloride (OPD)	Amber
Alkaline phosphatase	*p*-Nitrophenylphosphate-disodium salt (PNPP)	Yellow

General Procedure

- The sample with an unknown amount of antigen is immobilized on a solid support (usually a polystyrene microtiter plate) either nonspecifically (via adsorption to the surface) or specifically (via capture by another antibody specific to the same antigen, in a sandwich ELISA).
- After the antigen is immobilized, the detection antibody is added, forming a complex with the

antigen. The detection antibody can be covalently linked to an enzyme or can itself be detected by a secondary antibody that is linked to an enzyme through bioconjugation.

- Between each step, the plate is typically washed with a mild detergent solution to remove any proteins or antibodies that are nonspecifically bound.
- After the final wash step, the plate is developed by adding an enzymatic substrate to produce a visible signal, a color change in the substrate, which indicates the quantity of antigen in the sample.

Types of ELISA

Indirect ELISA

The indirect ELISA detects the presence of **antibody** in a sample (Fig 39.2):

- The antigen, for which the sample is to be analyzed, is adhered to the wells of the microtiter plate.
- The primary antibody, present in the sample, binds specifically to the antigen after addition of the sample.
- The solution is washed to remove unbound antibodies and then enzyme conjugated secondary antibodies are added.
- The substrate for enzyme is added to quantify the primary antibody through a color change.
- The concentration of primary antibody present in the serum directly correlates with the intensity of the color.

Advantages

- A wide variety of labeled secondary antibodies are available, commercially. Further, it is a versatile procedure because many primary antibodies can be made in one species and the same labeled secondary antibody can be used for detection. As a result of it, maximum immunoreactivity of the primary antibody is retained since it is not labeled.
- Sensitivity is increased because each primary antibody contains several epitopes that can be bound by the labeled secondary antibody, allowing for signal amplification.

  This, however, requires an extra incubation step, as a result of which cross-reactivity might occur with the secondary antibody, thereby resulting in nonspecific signal.

Sandwich ELISA

A sandwich ELISA is used to detect a specific sample antigen (Fig. 39.3). The steps are:

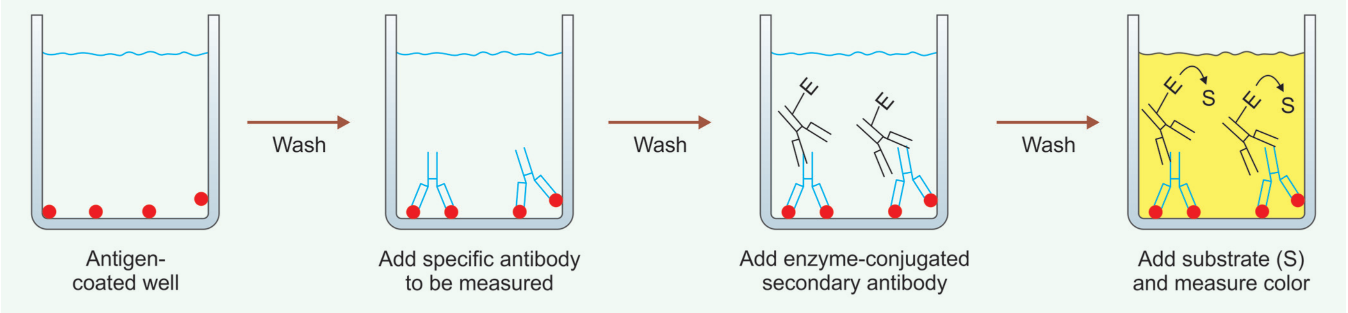

Wash
Wash
E
E
Wash
E
S
E
S
Antigen-
coated well
Add specific antibody
to be measured
Add enzyme-conjugated
secondary antibody
Add substrate (S)
and measure color

Fig. 39.2: Indirect ELISA

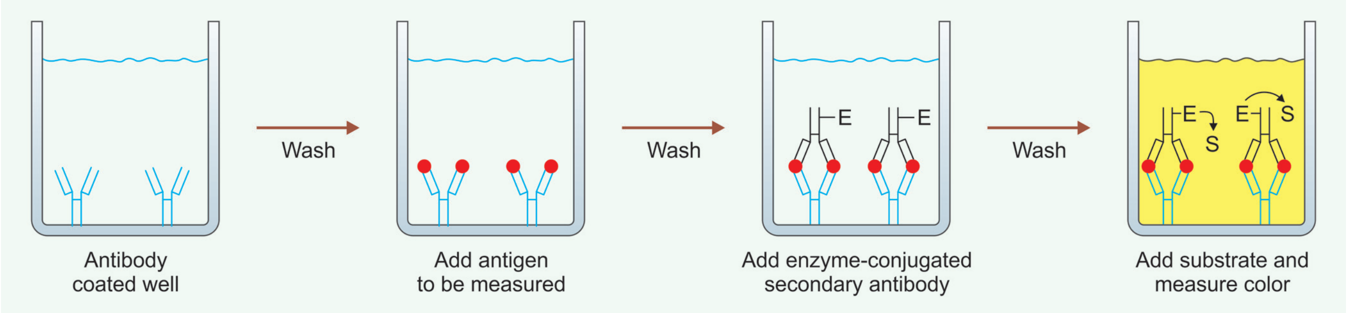

Wash
Wash
E
E
Wash
E
S
E
S
Antibody
coated well
Add antigen
to be measured
Add enzyme-conjugated
secondary antibody
Add substrate and
measure color

Fig. 39.3: Sandwich ELISA

- The wells of microtiter plate are coated with the antibodies.
- Nonspecific binding sites are blocked using bovine serum albumin.
- The antigen-containing sample is applied to the wells and captured by antibody.
- The plate is washed to remove unbound antigen.
- Enzyme linked secondary antibody is added, which, in turn, binds to antigen (**sandwich**, i.e. the antigen is stuck between the two antibodies).
- The plate is washed to remove the unbound antibody-enzyme conjugates.
- The substrate for enzyme is added to quantify the antigen.
- The absorbance of the chromogen is measured to determine the presence and quantity of antigen.

It has high specificity because the antigen is specifically captured and detected.

Competitive ELISA

This type of ELISA depends on the competitive reaction between the sample antigen and antigen bound to the wells of microtiter plate with the primary antibody (Fig. 39.4).

1. Unlabeled antibody is incubated in the presence of its antigen (sample).
2. These bound antibody/antigen complexes are then added to an antigen-coated well.
3. After an incubation, unbound antibodies are washed off, so unbound antibodies are removed. (The more antigen in the sample, the more Ag-Ab complexes are formed and so there are less unbound antibodies available to bind to the antigen in the well, hence **competition**).
4. Secondary antibody, specific to the primary antibody, is added. This second antibody is coupled to the enzyme.
5. A substrate is added, and remaining enzymes elicit a chromogenic signal.
6. The reaction is stopped to prevent eventual saturation of the signal.
7. Concentration of color is inversely proportional to the amount of antigen present in the sample.

It is highly sensitive even when the specific detecting antibody is present in relatively small amounts.

Some competitive ELISA kits include enzyme-linked antigen rather than enzyme-linked antibody. The labeled antigen competes for primary antibody binding sites with the sample antigen (unlabeled). The less antigen in the sample, the more labeled antigen is retained in the well and the stronger the signal.

High density microplates are, typically, used for screening applications, when throughput (number of samples processed per day) and assay cost per sample become critical parameters, with a typical assay volume between 5 and 50 µL per well.

ELISA READERS

ELISA reader is an instrument used for ELISA technique.

Types of ELISA Readers

1. Microplate ELISA readers
2. Automated ELISA readers

Microplate ELISA Readers

Microplate ELISA reader, also known as **microplate readers** or **microplate photometers**, are instruments which are used to detect biological, chemical or physical events of samples in microtiter plates. Common detection modes for microplate assays are absorbance, fluorescence intensity, luminescence, time-resolved fluorescence or fluorescence polarization.

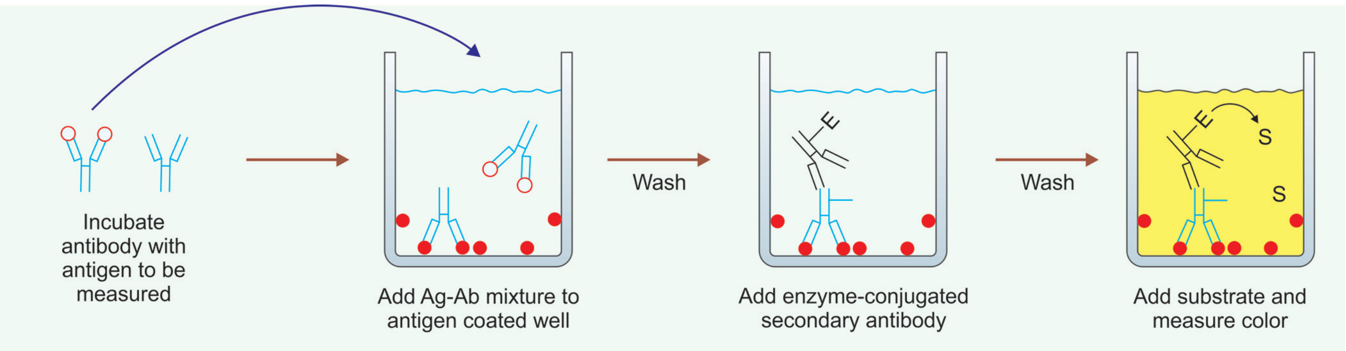

Incubate antibody with antigen to be measured
Add Ag-Ab mixture to antigen coated well
Wash
E
Add enzyme-conjugated secondary antibody
Wash
E
S
S
Add substrate and measure color

Fig. 39.4: Competitive ELISA

A light source illuminates the sample using a specific wavelength (selected by an optical filter or a monochromator) and a light detector, located on the other side of the well, measures how much of the light is transmitted through the sample. The amount of transmitted light is related to the concentration of the molecule of interest (Fig. 39.5).

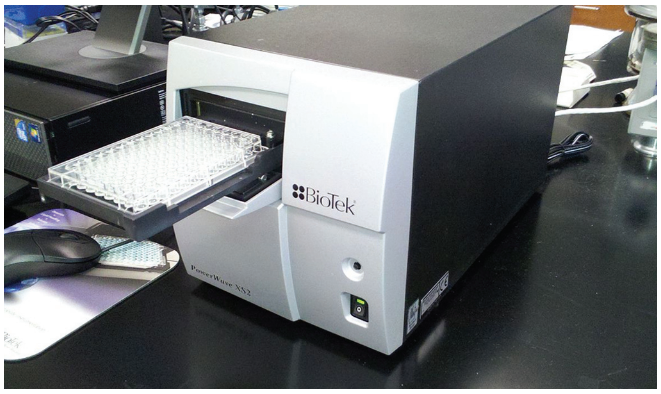


Fig. 39.5: A microplate reader with a 96-well microplate in the sample drawer

ELISA Plate

The test is done in an **ELISA plate**, also known as a **96-well plate** or **microplate** (8 × 12 matrix), with a typical reaction volume between 100 and 200 µL per well. It is the most common microplate format used in academic research laboratories or clinical diagnostic laboratories (Fig. 39.6).

Microplate ELISA readers are used for:

1. Homogeneous Enzyme Immunoassay

It is widely used in clinical analysis of hormones, drugs, etc. It is enzyme multiplied immunoassay technique (EMIT). It is carried out as follows:

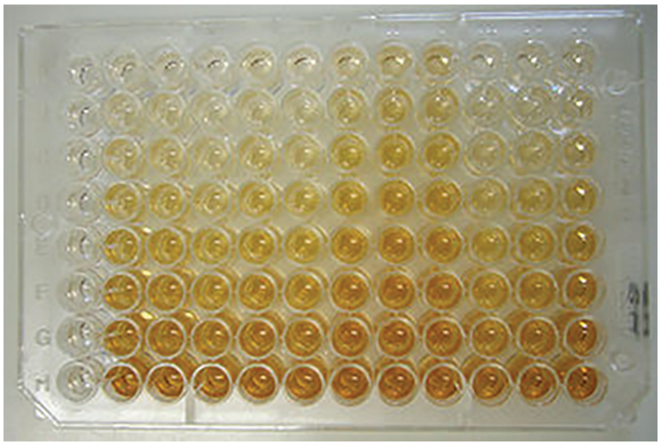


Fig. 39.6: ELISA plate

- Antibody against an analyte (hormone, drug or a metabolite) is added together with the substrate to the patient's sample.
- Binding of antibody and analyte occurs.
- An aliquot of enzyme conjugate of the analyte is added.
- Thereafter, enzyme-analyte conjugate binds with the excess of analyte antibody.
- This binding affects the enzyme activity by physically blocking access of the substrate to the active site of the enzyme or by changing the conformation of the enzyme, thereby altering its activity. The relative change in enzyme activity is proportional to the concentration of the analyte in the sample.

2. Heterogeneous Enzyme Assays

It constitutes:

- Sandwich ELISA
- Competitive ELISA

Automated ELISA Readers

These are used for:

1. Microparticle Enzyme Immunoassay (MEIA)

It uses a solution of suspended submicron latex particles to measure analytes. The particles are coated with the capture molecule, specific for the analyte being measured. MEIA is less time consuming since the effective surface area of microparticles increases assay kinetics and decreases assay incubation time. This is the automated methodology.

2. Fluorescence Polarization Immunoassay (FPIA)

It combines two technologies to determine analyte concentration, i.e.

- **Competitive protein binding**, and
- **Fluorescence polarization**

3. Radioactive Energy Attenuation Assays (REA)

It involves color development reactions. The presence of the analyte converts chromogen (unreacted dye) to a chromophore (colored dye). A stable fluorescent substance (fluorophore) is also added in the reaction mixture. The light absorbing property of chromophore produced, causes a decrease of measured fluorescent light intensity from the fluorophore, which is measured by FPIA optics assembly. Change in radiant energy intensity follows Beer's law. Production of chromophore is linked by the reaction system to the consumption of analyte, hence, development of fluorescence attenuation can be calibrated to measure the concentration of analyte in the sample.

Important Questions

1. What is ELISA? Describe its principle.
2. Describe various types of ELISA.
3. Write notes on:
    i. Types of ELISA readers
    ii. EMIT
    iii. Automated ELISA readers
    iv. Microplate ELISA readers

Chapter 40

Quality Control

The main objective of a laboratory is to provide reliable, timely and accurate test results. This is only possible through consistent monitoring and evaluation of the laboratory's performance and the implementation and follow-up of corrective actions for non-conformance to procedure. A reputation based on 10,000 good quality results is damaged by one poor quality result.

The issue of laboratory quality has evolved over more than 4 decades since the 1st recommendation for quality control was published in 1965. Laboratory quality control is designed to detect, reduce and correct deficiencies in a laboratory's internal analytical process prior to the release of patient results, in order to improve the quality of the results reported by the laboratory. It is a measure of precision or how well the measurement system reproduces the same result over time and under varying operating conditions.

Quality control in the clinical investigations is a statistical process used to monitor and evaluate the analytical process that produces patient results.

When a diagnostic test is performed in the medical laboratory, the outcome of the test is a result. The result may be a patient result or it may be a quality control (QC) result. The result may be quantitative (a number) or qualitative (positive or negative) or semi-quantitative (limited to a few different values).

QC results are used to validate whether the instrument is operating within pre-defined specifications, inferring that patient test results are reliable. Once the test system is validated, patient results can then be used for diagnosis, prognosis or treatment planning. For example, when a patient's serum is assayed (tested) for an analyte (a substance or constituent for which the laboratory test is conducted), e.g. glucose, the test result tells us how much glucose (concentration) is present in the blood. This result is then used by the physician to determine whether the patient has low, normal or high blood glucose. Let's assume the measured value of glucose in a patient's blood is 165 mg/dL (a unit of measure, milligrams per 100 mL). This result is abnormally high and indicates an inappropriate use of glucose by the body. But how does the person performing the test know that this result is truly reliable? It could be possible that the instrument is out of calibration and the patient's true glucose value is 95 mg/dL—a normal result. The question of reliability for most testing can be resolved by regular use of quality control materials and statistical processes.

Quality Control v/s Quality Assurance

Quality Control (QC)

QC is the set of operational techniques and activities that are used to fulfil requirements for quality. In healthcare testing, it is the set of procedures designed to monitor the test method and the results to assure test system performance.

QC includes testing control material, charting the results and analysing them to identify sources of error, and evaluating and documenting any remedial action taken as a result of this analysis.

Quality Assurance (QA)

QA refers to all those planned and systematic activities to provide confidence that the results given by the laboratory are correct.

The aim of quality control is simply to ensure that the results generated by the test are correct. However, quality assurance is concerned with much more, e.g. the right test is carried out on the right specimen, and that the right result and right interpretation is delivered to the right person at the right time. Thus, the purpose of QA is maintenance of the overall quality of patient results.

So, quality control is a vital part of quality assurance. All labs get benefit from quality control in terms of confidence in and reproducibility of test results.

QUALITY CONTROL PROCEDURES

- **Internal Quality Control** (IQC): It includes personnel, instrumentation, document control, reagent control and corrective actions.
- **External Quality Control** (EQC): External quality control is important to ensure that the laboratory is performing to an external standard.

How to Implement a QC Program?

- Establish written policies and procedures
- Assign responsibility for monitoring and reviewing
- Train staff
- Obtain control material
- Collect data
- Set target values (mean and SD)
- Establish control charts, e.g. Levey-Jennings chart
- Routinely plot control data
- Establish and implement troubleshooting and corrective action protocols
- Establish and maintain system for documentation

Factors that Affect the Test Results

Factors that affect the test results from the time of specimen collection and transport to result reporting include pre-analytical, analytical and post-analytical factors.

1. Pre-analytical Variables

Pre-analytical variability is defined as errors which occur when non-analytical factors change concentration of the analyte. These include:

- **Test to be conducted:** It should be ensured that the test performed is the same as requested.
- **Patient identification:** Labelling of specimen must be proper.
- **Turnaround time:** Should be kept to minimum.
- **Laboratory logs:** Entry of patient and test details should be made in lab registers and computers.
- **Transcription errors:** A substantial risk of transcription error exists from manual entry of data, even when results are double checked.
- **Patient preparation:** Proper patient preparation is essential to obtain meaningful test result.
- **Specimen collection:** It includes use of container and anticoagulant, time taken to send specimen to the laboratory, use of vacutainer tubes and laboratory personnel.
- **Specimen transport:** It is critical for some tests. Proper temperature control should be taken into account while transporting the sample from the collection area to the laboratory.
- **Specimen separation:** Includes monitoring the performance of the centrifuge and container used for storage.
- **Personnel:** When the tests are manually performed, there may be some variation from person to person.
- Safety precautions for all specimens must be observed.

2. Analytical Variables

- **Equipment reliability:** Equipment should meet technical needs, compatible, user and maintenance friendly, cost effective, validated procedural reliability using standard operating procedures.
- **Reagents stability, integrity and efficiency:** They should be stable, efficient, of desired quality, continuously available and validated.
- **Specificity and sensitivity:** Adequate sensitivity, sufficient specificity, cost effective, compatible with available infrastructure and expertise, interpretable and meets the needs/objectives.
- **Proficiency of personnel:** Education, training, aptitude, competence, commitment and adequate number. There should be continuous education, supervision and motivation.
- **Use of appropriate controls**:
  - **Internal:** May be prepared in lab and calibrated against national standards.
  - **External:** Supplied by manufacturer, national or international organization.
- **Documentation:** Policies, plans, procedures, instructions and records, quality control procedures and recorded test results involved in providing a service or the manufacture of a product, should be available in writing.

3. Post-analytical Variables

- Right recording and reporting of results
- Right interpretation with range of normal values
- Right turn around time
- Report to right user

External Quality Assessment

The control procedures described above monitor a single lab. To distinguish them from procedures used to compare the performance of different laboratories is known as external quality assessment (EQA).

External quality assessment (proficiency testing) is a program in which multiple specimens are periodically sent to members of a group of laboratories for analysis and/or identification, in which each laboratory's results are compared with those of the other laboratories in the group and/or with an assigned value, and reported to the participating laboratory and others.

The two procedures are complimentary. The internal QC is for daily monitoring of precision and accuracy, while the external QA is essential for long-term accuracy of analytical methods.

- EQA results evaluate performance of the laboratory against other laboratories participating in the same program.
- Different programs do this in different ways, e.g. t-test is used to test the statistical significance of any difference between individual labs observed mean and the group mean.
- When the difference is significant, the lab is alerted.
- Results are instrument and protocol specific.
- EQA results should be formally documented within the lab and should be available on request.

External Quality Assessment in India

In India EQA is done by Govt./private organizations. These include:

- **National Accreditation Board for Testing and Calibration Laboratories** (**NABL**), Dept. of Science and Technology, GOI (*http://www.nabl-india.org*)
- **ACBI-CMC External Quality Assurance Scheme, Christian Medical College**, Dept. of Clinical Biochemistry, **Vellore** (*http://www.cmcvellore.ac.in*)

It is to ensure that a laboratory practices and provides quality assurance for all phases of testing. The laboratory should:

- Enroll and satisfactorily participate in a performance evaluation/assessment program
- Establish reference range for analytes being tested
- Document training and competency assessment for their technicians
- Provide review and verification of all results released, including verbal result reports.

CALCULATION AND STATISTICS

Requirements for the Statistical Process

- **Regular testing of quality control products along with patient samples**
- **Comparison of quality control results to specific statistical limits (ranges)**

When a diagnostic test is performed in the medical laboratory, the outcome of the test is a **result**. The result may be a **patient result** or it may be a **quality control** (QC) **result**. The result may be **quantitative** (a number) or **qualitative** (positive or negative) or **semi-quantitative** (limited to a few different values).

QC results are used to validate whether the instrument is operating within pre-defined specifications, inferring that patient test results are reliable. Once the test system is validated, patient results can then be used for diagnosis, prognosis or treatment planning. For example, when a patient's serum is assayed (tested) for potassium, the test result tells us how much potassium (concentration) is present in the blood. This result is then used by the physician to determine whether the patient has low, normal or high potassium.

QC statistics for each test performed in the laboratory are calculated from the QC database, collected by regular testing of control products. The data collected is specific for each level of control. Consequently, the statistics and ranges calculated from this data are also specific for each level of control and reflect the behaviour of the test at specific concentrations. The most fundamental statistics used by the laboratory is the **mean**, **standard deviation** and **coefficient of variance** (Chapter 25).

The term **precision** is often used interchangeably with standard deviation. Another term, imprecision, is used to express how far apart numerical values are from each other. It is desirable to get repeated measurements of the same specimen as close as possible (**accuracy**).

Standard deviation is commonly used for preparing Levey-Jennings (L-J or LJ) chart.

Precision and Accuracy

Precision refers to the closeness of agreement between independent test results obtained under prescribed conditions. It is a measurement system related to reproducibility and repeatability, the degree to which repeated measurements under unchanged conditions show the same results.

Precision defines closeness of agreement between independent results of measurements, obtained under stipulated conditions.

- It is the dispersion of replicate measurements.
- Inter-assay with multiple runs, or intra-assay within a run.

- Repeatability (within same run).
- Reproducibility or closeness of results to each other, performed under changed conditions of measurement (time, operator, calibrators, reagent lot, etc.).
- Measured by the standard deviation or coefficient of variation

The **accuracy** of a measurement system is the degree of closeness of measurements of a quantity to that quantity's actual (true) value.

Although the two words precision and accuracy can be synonymous, they are deliberately contrasted in the context of the scientific method. A measurement system can be accurate but not precise, precise but not accurate, neither, or both. Eliminating the systematic error improves accuracy but does not change precision. A measurement system is considered valid if it is both, accurate and precise (Fig. 40.1).

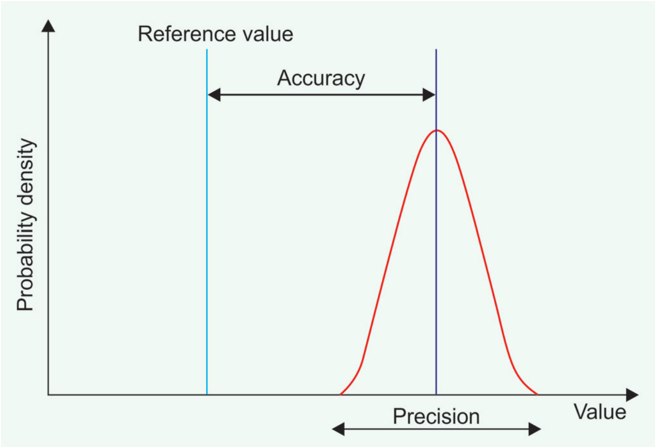


Fig. 40.1: Precision and accuracy

LEVEY-JENNINGS CHART

Levey-Jennings chart is a graph that identifies the mean, the working range and other limits for a control sample and shows results of control tests over a period of time. It is used to graph successive (run-to-run or day-to-day) quality control values (Fig. 40.2).

A Levey-Jennings chart is prepared for each test and level of control. The first step is to calculate decision limits. These limits are ±1s, ±2s and ±3s from the mean.

- When an analytical process is within control, approximately **68% of all QC values fall within ±1 standard deviation (1s)**.
- Likewise **95.5% of all QC values fall within ±2 standard deviation** (2s) of the mean. About 4.5% of all data will be outside the ±2s limits when the analytical process is in control.

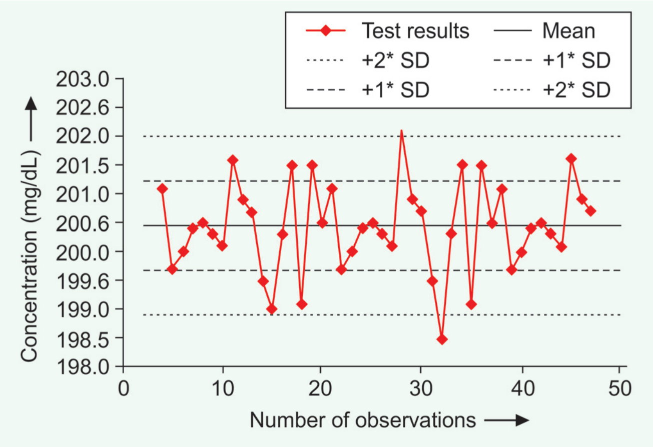


Fig. 40.2: Levey-Jennings chart

- Approximately **99.7% of all QC values are found to be within ±3 standard deviation** (3s) of the mean. As only 0.3%, or 3 out of 1000 points, will fall outside the ±3s limits, any value outside of ±3s is considered to be associated with a significant error condition and patient results should not be reported.

Using a Levey-Jennings Chart to Evaluate Run Quality

The laboratory needs to document that quality control materials are assayed and that the quality control results have been inspected to assure the quality of the analytical run. This documentation is accomplished by maintaining a QC log and using the Levey-Jennings chart on a regular basis.

The QC log can be maintained on a computer or on paper. The log should identify the name of the test, the instrument, units, the date the test is performed, the initials of the person performing the test, and the results for each level of control assayed. Optional items for the log include: method and the assay temperature (usually included for enzymes).

There should be room to write in actions taken to resolve any situation which is identified as "out-of-control" or unacceptable and a place for documentation of supervisory review.

Once the QC results are entered into the QC log, they should be plotted on the Levey-Jennings chart. When the results are plotted, an assessment can be made about the quality of the run. The technologist/technician performing the test should look for systematic error and random error.

Systematic Error

Systematic error refers to a trend or shift away from the laboratory mean. It is evidenced by a change in the mean of the control values. The change in the mean may be gradual and demonstrated as a **trend** in control values or it may be abrupt and demonstrated as a **shift** in control values.

Trend

Trend is a gradual, often subtle, increase or decrease in control values and possibly patient values. It is a type of systematic error. A trend indicates a gradual loss of reliability in the test system. Trends are usually subtle. Causes of trending may include:

- Deterioration of the instrument light source
- Gradual accumulation of debris in sample/reagent tubing
- Gradual accumulation of debris on electrode surfaces
- Aging of reagents
- Gradual deterioration of control materials
- Gradual deterioration of incubation chamber temperature (enzymes only)
- Gradual deterioration of light filter integrity
- Gradual deterioration of calibration

Shift

Shift means a sudden and eventually stable change in control values and possibly patient values. It is a type of systematic error. Shifts in QC data represent a sudden and dramatic positive or negative change in test system performance.

Shifts may be caused by:

- Sudden failure or change in the light source
- Change in reagent formulation
- Change of reagent lot
- Major instrument maintenance
- Sudden change in incubation temperature (enzymes only)
- Change in room temperature or humidity
- Failure in the sampling system
- Failure in reagent dispense system
- Inaccurate calibration/recalibration

Random Error

Technically, random error is any deviation away from an expected result. For QC results, any positive or negative deviation away from the calculated mean is defined as random error. There is acceptable (or expected) random error as defined and quantified by standard deviation. There is unacceptable (unexpected) random error that is any data point outside the expected population of data (e.g. a data point outside the ±3s limits).

WESTGARD RULES

Westgard rules refer to a set of 6 statistical rules with multiple applications when used separately or in concert with each other that are used to verify the reliability or lack of reliability for patient test results.

In 1981, James Westgard of the University of Wisconsin published an article on laboratory quality control that set the basis for evaluating analytical run quality for medical laboratories. The elements of the Westgard system are based on principles of statistical process control used in industry nationwide since the 1950s. There are six basic rules in the Westgard scheme. These rules are used individually or in combination to evaluate the quality of analytical runs.

Westgard devised a shorthand notation for expressing quality control rules. Most of the quality control rules can be expressed as NL where N represents the number of control observations to be evaluated and L represents the statistical limit for evaluating the control observations, e.g. 1_{3s} represents a control rule that is violated when one control observation exceeds the ±3s control limits.

Rule 1_{2s}

This is a warning rule that is violated when a single control observation is outside the ±2s limits. Remember that in the absence of added analytical error, about 4.5% of all quality control results will fall between the 2s and 3s limits. This rule merely warns that random error or systematic error may be present in the test system. The relationship between this value and other control results within the current and previous analytical runs must be examined. If no relationship can be found and no source of error can be identified, it must be assumed that a single control value outside the ±2s limits is an acceptable random error. Patient results can be reported.

Rule 1_{3s}

This rule identifies unacceptable random error or possibly the beginning of a large systematic error. Any QC result outside ±3s violates this rule.

Rule 2_{2s}

This rule identifies systematic error only. The criteria for violation of this rule are:

- Two consecutive QC results
- Greater than 2s
- On the same side of the mean

There are two applications to this rule: Within-run and across runs. The within-run application affects all control results obtained for the current analytical run. For example, if a normal (level I) and abnormal (level II) control are assayed in this run and both levels of control is greater than 2s on the same side of the mean, this run violates the within-run application for systematic error. If, however, level I is -1s and level II is +2.5s (a violation of the 1_{2s} rule), the level II result from the previous run must be examined. If level II in the previous run was at +2.0s or greater, then the across run application for systematic error is violated. Violation of the within-run application indicates that systematic error is present and that it affects potentially the entire analytical curve. Violation of the across run application indicates that only a single portion of the analytical curve is affected by the error.

Rule 2_{4s}

This rule identifies random error only and is applied only within the current run. If there is at least a $4s_{difference}$ between control values within a single run, the rule is violated for random error. For example, assume both level I and level II have been assayed within the current run. Level I is +2.8s above the mean and level II is -1.3s below the mean. The total difference between the two control levels is greater than 4s (e.g. [+2.8s – (-1.3s)] = 4.1s).

Rule 3_{1s}

The criteria which must be met to violate this rule are:

- Three consecutive results
- Greater than 1s
- On the same side of the mean

Rule 4_{1s}

The criteria which must be met to violate this rule are:

- Four consecutive results
- Greater than 1s
- On the same side of the mean

QUALITY CONTROL PRODUCTS

A quality control product (control material) is a patient-like material, ideally made from human serum, urine or spinal fluid. A control product can be a liquid or lyophilized (the characteristic describing the result of the process of vacuum-freeze-drying a liquid material to make its components more stable) material, and is composed of one or more constituents (analytes) of known concentration. Control products should be tested in the same manner as patient's samples.

A quality control product usually contains many different analytes. For example, a general chemistry control can contain any number of chemistry analytes, including potassium, glucose, albumin, calcium, etc.

A **normal** control is a product that contains physiologically normal concentration of a particular analyte.

An **abnormal** control is a a control product which contains a physiologically high or low concentration of a particular analyte. For example, the normal range for blood glucose level is about 60–100 mg/dL. A normal control would contain glucose at a level within this range. An abnormal control would contain glucose at a level below 60 mg/dL or above 100 mg/dL.

Regular Testing

Good laboratory practice requires testing normal and abnormal controls for each test at least daily to monitor the analytical process. If the test is stable for less than 24 hours or some change has occurred which could potentially affect the test stability, controls should be assayed more frequently. Regular testing of quality control products creates a QC database that the laboratory uses to validate the test system. Validation occurs by comparing daily QC results to a laboratory-defined range of QC values. The lab-defined range is calculated from QC data collected from testing of normal and abnormal controls.

Characteristics of the Quality Control Material

1. The quality control material should be, approximately, the same matrix as patient's specimen, taking into account the properties such as viscosity, turbidity, composition and color.
2. It should be simple to use, with minimal vial to vial variability.
3. It should be stable for long period of time and available in large quantity, for a single batch, to last at least for one year.
4. Liquid controls are more convenient to use than the lyophilized controls because they have not to be reconstituted, thus, minimizing the pipetting error.

Laboratory quality control material should be run in the beginning of each shift, after an instrument gets serviced, when reagent lots are changed, after calibration and whenever patient's result seems to be inappropriate.

Criteria for the Selection of a Quality Control Product

Many quality control products are available for laboratories and choosing the right quality control product requires careful consideration. Sometimes, laboratory decision makers yield to the temptation of purchasing the most inexpensive product. It may be due to the reason that cheaper alternatives have certain limitations. For example:

Shelf Life

Shelf life refers to the amount of time an unopened control product is considered reliable when stored properly. While purchasing a quality control product, it is essential to know the approximate volume of control to be used each day. For example, routine chemistry control products are usually sold in 10 mL vials. Thus, laboratories, which use 10 mL or more per day, generally are not concerned with its stability but for the laboratories that use a low volume of control (e.g. 1 mL/day), shelf life becomes an important issue. The short shelf life, thus, can result in unnecessary waste, if the laboratory cannot use all the material.

Box Pricing

Box pricing is a misleading quoting practice and many laboratories fall into the trap, at one time or another. For example, a laboratory is negotiating price with two vendors for an expensive quality control product. One vendor offers the product at Rs 10 per mL or Rs 1000 per box while the other vendor offers the product at Rs 800 per box without quoting a per mL price. The first vendor provides 10 × 10, i.e. 100 mL for Rs 1000, while the second vendor provides only 50 mL for Rs 800. Accordingly, the product cost per mL from the second vendor is equal to Rs 16 per mL. Thus, always compare the quality control product quotes on a per mL basis and not box price.

Nature of the Product

The product may not be similar to the patient's specimen (serum, urine, spinal fluid or plasma). This, in turn, can cause a problem with certain tests, such as it may not interact with the test system in the manner, the patient's sample reacts.

Some inexpensive quality control products may not have all analytes, at clinically relevant decision levels.

Types of Control Materials

Assayed

Mean calculated by the manufacturer—must verify in the laboratory.

Unassayed

Less expensive—must perform data analysis.

Homemade or in-house

Pooled sera collected in the laboratory is characterized and preserved in small quantities for daily use.

Managing Control Materials

- Sufficient material from same lot number or serum pool for one year's testing
- May be frozen, freeze-dried or chemically preserved according to instructions by the manufacturer
- Requires very accurate reconstitution, if this step is necessary
- Always store as recommended by manufacturer.

Calibrators

- Has a known concentration of the substance (analyte) being measured.
- Used to adjust instrument, test kit, system in order to standardize the assay.
- Sometimes called a standard, although usually not a true standard.
- This is not a control.

Sometimes control products are not human. Control products can be of animal origin, aqueous solution or a commercially prepared organic matrix.

Specimens that are analyzed for QC purpose are known as control materials.

- Should have preferably the same matrix as the specimen.
- Control materials should be available in a stable form, in aliquots or vials, for analysis over an extended period of time, at least 1 year.
- Minimal vial-to-vial variation should exist.

In India, medical laboratories can volunteer for accreditation of one or more services offered by them. The **National Accreditation Board for Testing and Calibration Laboratories** (**NABL**) has been providing accreditation services to medical laboratories since 1998 and is currently following ISO 15189; 2007 standards.

The proposed ICMR guidelines for Good Clinical Laboratory Practices (GCLP) aim to elucidate step wise procedures which should be followed by laboratories to strengthen the quality of test results.

PRACTICAL EXERCISE

1. Prepare Levey-Jennings chart for blood glucose in your lab.

Important Questions

1. What is quality control? Describe various types of quality control procedures for a clinical chemistry lab.
2. Describe various factors affecting clinical chemistry test results.
3. Differentiate between:
    1. Internal and external quality control
    2. Accuracy and precision
    3. Quality control and quality assurance
    4. Pre-analytical and analytical variables
4. Write notes on:
    1. Quality control
    2. Levey-Jennings chart
    3. Westgard rules
    4. Quality control materials

Chapter

41

Automation in Clinical Chemistry Laboratory

The word **automation** is inspired by the word automatic, means exercising control without interference. Thus, it refers to work getting done by machines, which can run on their own, without continuous monitoring. This, in turn, reduces our workload and need for continuous supervision.

Automation in clinical chemistry describes use of laboratory instruments and specimen processing, to perform clinical laboratory assays, with minimal involvement of the technologist.

Development of automated instruments for clinical laboratory began in early 1950s, which enabled laboratory to process much of its workload in a lesser time and more efficiently. Major benefit of automation is reduction in variability of results (errors of analysis), by eliminating the jobs, which are repetitive and monotonous for men, and can predispose to errors. Improved reproducibility and trueness of results, by skillfully designed automated instruments, has led to significant improvement in the quality of lab tests.

Steps in the Analysis Process in a Laboratory

There are several **steps in the analysis process in a laboratory**, such as:

1. Identification of the patient
2. Getting the correct sample for the desired test
3. Identification and proper labelling of the sample
4. Delivery of the sample under proper conditions and within time
5. Preparation of the sample for the desired test
6. Sample loading/aspiration
7. Analysis
8. Entry in the register, and
9. Reporting

Advantages of Automation in Clinical Chemistry Laboratory

1. Increase in the number of tests performed by the laboratory in a given period of time. The labor component, devoted to any single test, is also minimized. This, in turn, effectively lowers the cost per test.
2. Minimal variation in results from one laboratory to another. By reproducing the components in a procedure as identically as possible, the coefficient of variation is lowered and reproducibility is increased. Automation, however, cannot correct for deficiencies inherent in methodology.
3. Automation eliminates potential errors of manual analysis, such as pipetting, calculation and transcription of results.
4. Instruments use small amount of sample and reagent(s). This, in turn, requires less blood to be drawn from the patient. In addition, use of small amounts of reagents decreases cost of consumables.

TOTAL LABORATORY AUTOMATION

This is a large-scale automation that includes mostly all the pre-analytical automation functions, i.e. transportation of patient's specimens from conveyer belt to various analytical stations, centrifugation of blood specimens, aspiration of serum from centrifuged tubes into aliquots tubes, decapping of tubes, bar code labelling of aliquot tubes and sorting of tubes according to the lab destination.

STEPS IN TOTAL AUTOMATED ANALYTICAL PROCESS

Sample Collection

Use of glucometer is one such example where just by pressing a button on finger-tip, the finger is pricked with least pain and blood glucose analysis is also done then and there. However, not all tests can be done with glucometer.

Automation in sample collection, mainly, refers to improved, fast and least discomfort causing techniques of collection, such as robotic system, vacutainers, etc.

Use of Vacutainer

Here the phlebotomist need not pull the syringe, blood gets sucked in due to negative pressure filling the vacuum. Different types of vacutainer for serum collection and for plasma collection, using different types of anticoagulants, are available. The identification is done with the color of caps used (Chapter 15).

Sample Identification

This is an important link between patient's identity and specimen. The techniques included are labelling, bar-coding, optical character recognition, magnetic stripes and magnetic ink character recognition, optical mark readers, and smart cards.

Labelling is one of the oldest methods while bar-coding is most frequently used. It first generates a unique identity or hospital number for each new patient. A record is maintained thereafter for him/her. All samples collected have to bear the name and details of the patient along with this unique identity (hospital number). The same is used while entering the details into auto analyzer software and result is also published with this number and other details.

Bar-coding

This is one of the major advancements in the automation of specimen identification. A bar-coded label, generated by the laboratory information system, bearing the specimen accession number, is placed on the specimen container. This, in turn, is read by one or more bar-code readers placed in the analytical system. This information is then subsequently read by the system software. It has the advantage, that it can be scanned and read by bar code reader accurately, so transcriptional error (mistake in writing manually) is avoided. Bar coding of the patient, unique sample identity is attached to his/her wrist. Even when patient is sleeping or unconscious, nurse can read the code for patient identification (Fig. 41.1).

Advantages of Bar-code System

1. Elimination of making work/load list for the system.
2. Prevention of mix-ups in the placement of tubes during sampling.
3. Analysis of specimen in defined sequence.

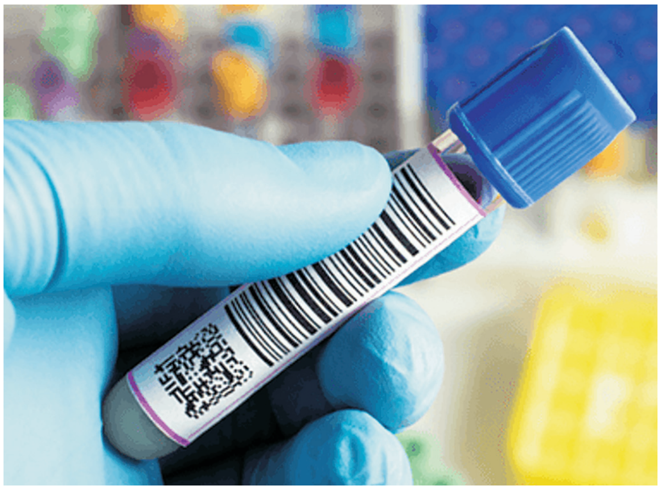


Fig. 41.1: Bar-coded tubes for use in sample collection

Sample Delivery to the Laboratory

Several methods can be used. These include courier service, pneumatic tube system, electric track vehicles and mobile robots.

Most of the laboratories rely on human pick up system or conveyer belt system. Though, cheaper, it may lead to human error, delays, etc. Pneumatic tube systems (use of pressurized gas to move the tubes containing samples) are used in some laboratories. It provides rapid transport system as well as is cost effective. It is being used in many hospitals and provide a safe method of transport for the sample. However, care has to be taken that acceleration and deceleration should not damage any sample. In very advanced laboratory, mobile robots are also used.

Sample Preparation

Most of the labs depend on technicians for sample processing (such as serum separation) as soon as sample arrives. However, introduction of automation can reduce the workload on technicians and save their time and expertise for analysis purpose. Therefore, nowadays many semi-automated devices are developed, which can analyse whole blood itself. For example, automated ion selective electrode, use of dry chemistry, etc.

The automated sample processors can be:

- Stand-alone sample processor, and
- Modular sample processor.

These automated sample processors can do the following tasks:

- Sorting of samples
- Removing caps

- Separating samples
- Bar coding, etc.

An automated sample processor can solve the task of bar coding and sample delivery via conveyer belt system.

Sample Analysis

1. Specimen Loading and aspiration

Most instruments use either circular carousels or rectangular racks as specimen containers for holding disposable sample cups or primary sample tubes in the loading or pipetting zone of the analyzer.

Many state-of-the-art analyzers (e.g. CX/LX SERIES, dimension) directly take sample from primary collection tubes of various sizes.

The tray or racks move automatically at predetermined speed to various positions and the speed determines the number of specimens analyzed per hour. In some analyzers specimen may be added continuously by the operator, as and when the specimens become available. This is a very advantageous feature of automation to insert new specimen ahead of the specimens already placed in the loading zone. This unique feature allows timely analysis of specimen with a high medical priority, immediately after they are received in clinical laboratory STAT sample (Fig. 41.2).

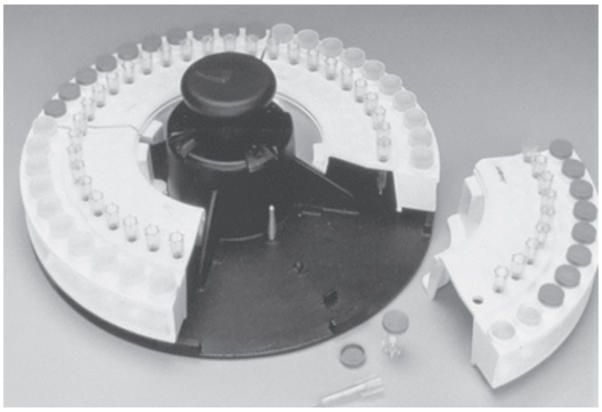


Fig. 41.2: Circular carousel for holding disposable sample cups (Vitros autoanalyzer with four quadrant trays, each for holding ten samples, that fits on a tray carrier)

2. Specimen Delivery within the Analyzer

The method of specimen delivery within the analyzer is a major difference between continuous-flow system and a discrete analyzer.

In continuous flow analyzer, specimen is aspirated through the sample probe into a continuous reagent stream, air is aspirated for a specified time to produce a bubble in between sample and reagent plugs of liquid. Then the probe descends into a container where wash solution is drawn into the probe and through the system.

On the other hand, in discrete analyzer the specimen is aspirated into the sample probe and then delivered through the same orifice into the reaction cuvette.

These chemistry analyzers use computer-controlled stepping motors to drive both the sampling and washout syringes. After every few seconds, the sampling probe enters a specimen container, withdraws the required volume, moves to the cuvette and dispenses the aliquot with a volume of water to wash the probe. The washout volume is adjusted to yield the final reaction volume. If a procedure's range of linearity is exceeded, the system will retrieve the original sample tube, repeat the test using one-fourth of the original sample volume for the repeat test and calculate a new result, taking the dilution into consideration.

Carryover

It is defined as the unintended transfer of a quantity of analyte or reagent by an analytical system from one specimen into the next one. This introduces error into the analytical result and should be minimized. This has been reduced in many systems by flushing the external and internal surfaces of the sample probes with copious amounts of diluents or the use of disposable reaction vessels and measuring cuvettes. To avoid the carry over through pipetting system, many systems have adopted disposable sample-probe tips (Vitros analyzers).

3. Reagent Handling and Storage

Most automated systems use liquid reagents, stored in plastic containers with proper refrigeration system inside the analyzer only that maintain the temperature at 4° to 10° C (e.g. Synchron CX, LX Dimension).

Many automate systems use nonliquid reagents (e.g. Vitros). For the color reactions, this system uses multilayered square slides in which reagent dispersed, are activated by the diffusion of the specimen fluid into the layers.

4. Reagent Identification

Reagent containers are labeled that give the information pertaining to the reagent identification, volume of the contents or the number of tests for which the contents can be used, expiration date and lot number. Nowadays, a bar-code label is put on the container and manufacturer is able to retrieve any information from the bar-code, when necessary.

In immunoassays, bar-code also contains the key information about the multiple calibrators and values of calibrators constants, which ensures that calibrator functions are properly integrated with the analytical process.

5. Chemical Reaction Phase

This phase occurs when aliquots of specimen and reagents are allowed to react, chemically. This depends on the following factors:

1. Vessel in which the reaction occurs and the cuvette in which the reaction is measured.
2. Timing of the reaction.
3. Mixing and transport of reactants
4. Thermal conditioner of the fluids.

Types of reaction cuvettes

As already mentioned above, in continuous flow system each specimen is passed through the same continuous stream and is subjected to the same analytical reaction as every other specimen and even at the same rate. So, the reaction occurs in the tube that can serve both, as a flow container as well as cuvette.

In discrete system, each specimen has its own physical and chemical space, separate from every other sample. They have following unique features:

1. Disposable or reusable reaction vessels (such as in Synchron CX series, AU 400 and AU 600 Abott series) are transported through the system, after specimen and reagents have been dispensed.
2. A cuvette is either disposable or permanent. The use of disposable cuvettes has simplified automation and eliminated the carry over things.

Automation in Cuvette Cleaning

1. The cleaning sequence involves aspiration of the reaction mixture out of the cuvette at wash station.
2. The detergent, alkaline or acid wash solution, is repeatedly dispensed and aspirated out of the cuvette.
3. Rinsed several times with deionized water.
4. Dried by vacuum or pressurized air.
5. Optical clarity is verified automatically.
6. If performance is unsatisfactory, the operator is being alerted for the replacement of the cuvette. Till they are changed, the faulty cuvettes will be flagged.

Wash stations on a chemistry analyzer perform the following:

1. Aspirate reaction waste and dispense water
2. Aspirate and dispense rinse water
3. Aspirate rinse water and dispense water for measurement of cell blank, and
4. Aspirate cell blank water to dryness (Fig. 41.3).

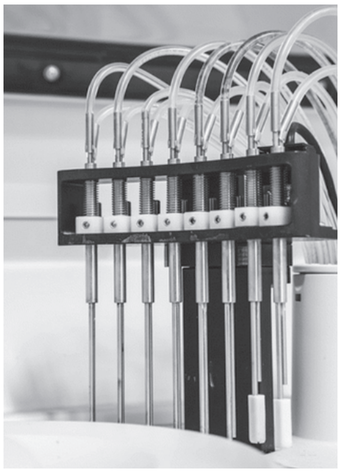


Fig. 41.3: Cuvette cleaning (Boehringer Mannheim autoanalyzer)

The Timings of the Reaction

In discrete analyzers, specimen and reagents are added to a cuvette in a timed sequence and absorbance readings are taken at particular intervals (depending on the parameter).

Since the reaction rate is controlled by temperature, the reagent timing and spectrophotometric functions must be coordinated to work in harmony with the chosen temperature. The environment of the cuvettes is maintained at a constant temperature by a liquid bath containing water or some other fluid with good heat transfer properties, in which the cuvettes move.

Mixing of Reactants

This is one of the vital steps for any reaction. In a continuous flow, the coiled tubes serve the function of proper mixing. The other techniques being used in various automated analyzers include forceful dispensing, magnetic stirring, rotating paddle or use of ultrasonic energy (Fig. 41.4).

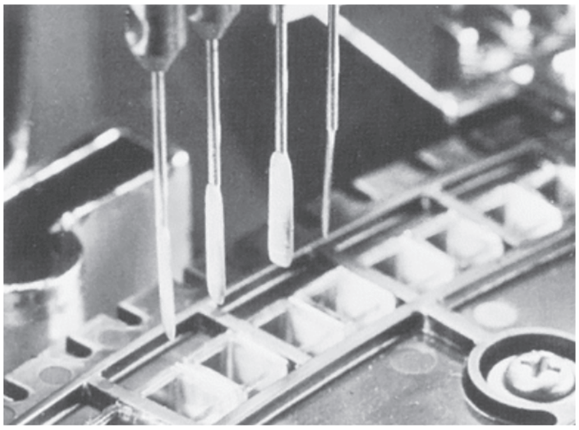


Fig. 41.4: Stirring paddles (Boehringer Mannheim autoanalyzer)

Thermal Conditioner of the Fluid

Air bath, water bath and contact with a heating or cooling plate are used for thermal regulation.

Measurement Approaches

The following techniques are being used in most of the automated analyzers:

1. Photometry
2. Spectrophotometry
3. Reflectance photometry
4. Fluorometry
5. Immunoassay—chemiluminescence and electro-chemiluminescence
6. Ion-selective electrode
7. Turbidimetry and nephelometry

Signal Processing, Data Handling and Process Control

Computers and computer telecommunications are the integral part of the entire analytical and reporting process and it controls the data input and data reporting in automated analyzers.

AUTOMATION INSTRUMENTS IN A CLINICAL BIOCHEMISTRY LABORATORY

Depending upon the work load, the instruments required in a clinical biochemistry laboratory, include **clinical chemistry auto-analyzers**; **ion selective electrodes (ISE)** for electrolytes (Chapter 24); facilities for hormonal assays, such as enzyme immune assay (**ELISA**; Chapter 39) and **radioimmunoassay** (RIA; Chapter 50); **chemiluminescence** and **high-performance liquid chromatography** (HPLC; Chapter 37) for various specialized analytes; and **atomic absorption spectrophotometer** for minerals, etc.

AUTOANALYZERS

For routine clinical chemistry investigations, various types of autoanalyzers are available in the market, which may be grouped into different categories, as:

Modular vs Integrated Systems

Modular System of Autoanalyzer

It can be compared with the modular kitchen, where many small cabinets are fitted according to its use. So, if there is a problem with any part, only that part can be rectified/replaced without disrupting rest of the kitchen.

Similarly, in a lab when whole automated system is subdivided into multiple useful parts, it becomes a modular designed automation. Each part is created separately in such a way that they can fit into different systems. It is useful in a way, because if one part of the machine goes out of order, the same can be replaced without affecting the working of the entire machine.

So, in a **modular system**, an autoanalyzer is designed by assembling different functional parts, together. This, in turn, increases flexibility of the machine, according to the customer's demand, e.g. **ion selective electrode**. Due to modular approach, facility for analysis of sodium, potassium and chloride can be built into the system or added later, as a module, e.g. Roche Modular P autoanalyzer.

Integrated System of Autoanalyzer

In the integrated system, the entire equipment is designed in such a way that every component forms an essential part of the machine. Basically, it is due to merging of the functionality of the different softwares into an integrated solution.

It offers some advantages, such as improved communication and data exchange between different softwares and the machine. However, it also has disadvantages, e.g. service and maintenance of the machine may be difficult and expensive, since we have to depend on the company's engineer, even for a minor problem.

Open vs Closed Systems

Another way of classifying automated analyzers is categorizing them as open and closed systems.

Open System

In this system, the operator has the advantage that he can purchase reagents from any company. Alternatively, one can also prepare in-house reagents. So, we can save money by buying reagents which are cheaper, thereby, reducing cost per test. The operator can also change the parameters related to analysis.

Closed System

In this system, operator has to buy chemicals from the particular company only, because reagents from other companies will not be accepted by the machine and the machine will not run. Accordingly, cost per test increases. Furthermore, reagents have to be provided in unique containers or format only, as prescribed by the manufacturer. This too adds to the cost per test. However, it allows high degree of automation and the laboratory can be managed by just one or two well-trained technical assistants only.

Some of the differences between open and closed systems are given in Table 41.1.

Table 41.1 Differences between open and closed systems of auto-analyzers

Open system	Closed system
1. In-house reagents or reagents from different suppliers can be used	Reagents in a unique container or format are provided by the manufacturer
2. Reagents are less expensive	Proprietary components are relatively expensive
3. Reconstitution of reagents is required	Reconstitution or preparation does not require technologist's involvement.

According to the Type of Processing

Continuous Flow Processing

A continuous flow analyzer (CFA) has following components:

- Sampler
- Pump
- Mixing coil
- Heater/incubator
- Sample treatment chamber (dialysis, distillation, etc.)
- Signal detector
- Read out device (data generator)

This provides one analysis per analyte for one sample at a time. The flowing carrier solution passes through small tubes continuously. This is the main principle of continuous flow processing. Here, sample is injected into a flowing carrier solution. The sample mixes with diluents and reagent, and is sent through the tubing and mixing coils. The machine prevents carry over effect between different samples by injecting bubbles of air. The air bubbles literally create separate space for different reactions to take place inside the tubing and mixing coils. The tubing passes the samples from one apparatus to the other. There is different apparatus for different functions, such as ion exchange, heating, incubation and finally recording of the signal.

The flow conditions are regulated. When reaction is taking place, the optical density of the color formed is read and results are obtained. So, we do not have to wait till the reaction ends.

It has separate heater for promoting enzymatic reaction and color development. For example, analysis of total protein, albumin and creatinine in a patient of nephrotic syndrome. In case of continuous flow processing analyzer, the patient sample will be sucked by the instrument and injected into the tubing with reagents for protein, and diluents (if needed). Next, air bubbles will be injected and patient sample will be sucked again. This time instrument will inject reagent for albumin. Mixing will be done inside the tubing and mixing coils. Again, the process will be repeated for creatinine estimation. The reactions for 3 tests will occur inside the same long tubing but will remain separate due to air bubbles in between.

As the sample and standard are treated in the same manner, mixed in same condition, travel the same length of tubing, it removes the difference between the two. So, the difference in reading for test tube from that of standard gives the value. Even though originally, CFA was designed to process only colorimetric reactions, later on CFA were designed to read reactions based on ion selective electrode, flame photometry, fluorometry, etc., depending on the need of the laboratories.

Its major use is for batch analysis, such as liver profile, lipid profile, renal profile, etc.

There are certain disadvantages in this system. Even when there is no test to be done, reagents are drawn to maintain the flow. This adds to the cost per test.

- Maintenance of instrument is required more frequently.
- The probe and internal tubing must be free of clogs. When there is no sample the probe must be dipped in distilled water to avoid blockage or precipitation.
- The machine itself occupies large space.

Discrete Analysis

In contrast to continuous flow analysis, discrete analyzers are designed for each specimen in a batch, its own physical and chemical space, and separate from every other specimen.

In this type of autoanalyzer, each sample is provided a discrete space. It means each analysis even for same analyte or sample takes place at different cups. This is the main principle of discrete processing.

For example, in a patient of nephrotic syndrome where we have to analyse total protein, albumin and creatinine. In discrete processing analyzer, the same patient sample will be sucked by the instrument and poured into 3 different cups. Then reagents for protein, albumin and creatinine, and diluents (if needed) will be added. Mixing will be done. Cups will be read at different times to give results.

Here, exact amount of sample and reagent is aspirated and mixed. So, there is no loss of excess reagents used for flow, as in continuous flow processing. As each analysis is done in different cups and read in different cuvettes, there is no carry over effect. So literally, each analysis is discrete from other.

- This is more useful
- Saves reagent cost and hence popular than continuous flow analyzers.
- No sample carry over effects

Types of discrete analyzers

There are different types of discrete analyzers, of which two major examples are:

- Centrifugal analyzers, and
- Random Access analyzers

Centrifugal Analyzers

Here the discrete aliquots of specimen and reagents are pipetted into discrete chambers. Specimens are analyzed in parallel by spinning the rotor and using the centrifugal force to transfer and mix the aliquots of specimens and reagents into radially located cuvettes. With the help of integrated computer system, multiple absorbance readings are taken from each cuvette.

This is time saving for batch analysis because all cuvettes can be read at a time. But its disadvantage is that only one test can be performed at a time.

Random Access Analyzers

Each sample can be analysed for multiple tests. Further, multiple samples for one test also can be done by giving appropriate command to computer software. It is the most versatile of all types of analyzers. For example, if we have 3 different samples out of which first one needs renal profile, second one needs only glucose and urea, and the third one needs triglyceride, albumin and calcium. So, the person has to take 3 different sample cups and load 3 samples. Then he has to enter sample number, cup number and the tests required. And when he presses the start button tests are done automatically.

Unique features of these analyzers are as follows:

1. They perform analysis on a batch of specimens sequentially, with each specimen being analyzed for a different selection of tests. The tests performed are selectable through the use of different containers of liquid reagents or different reagent packs.
2. The groups of tests or profiles requested for any sample are defined at the time the requested samples are entered into the analyzer by means of keyboard or by the lab information system using bar coding on the specimen tube.
3. They are able to take multiple number and variety of analytes in each specimen. This is possible because of the ability to take multiple absorbance or reflectance measurements for each assay.
4. The most unique property of these analyzers is enough self-diagnosis, features are programmed in analyzer software and this allows the technologist, operating the instrument, to leave the instrument for a short period of time. Further, more than one analyzers can be operated at a time.

Examples include, Abbott Diagnostics Aeroset, Beckman Coulter Synchron, Dade Behring Dimension, Roche Cobas Integra, Roche Diagnostics Modular Analytics P Module.

DRY CHEMICAL ANALYZERS

Dry chemical methods utilize **dry slides** that are **composed of several layers,** which may include:

1. Spreading layer
2. Scavenger layer
3. Reagent layer(s)
4. Plastic or support layer

The reagent layer(s) contain enzymes, dry precursor and buffers necessary for the analysis of an analyte. **Sample, control or standard is deposited on the spreading layer.** Based on the test, selected components are allowed to penetrate to the reagent layer(s), which in turn activate the dry/dehydrated reagents. A **chemical reaction gets initiated to produce a color. Light is passed beneath the support layer** and is directed through the reagent layer(s). As the light hits the white spreading layer, **some of the light reflects back** through the reagent layer(s) **to a photocell.** The amount of reflected light, which is **proportional to the color intensity,** is used to determine concentration of the analyte.

It also has a **disadvantage** that samples with abnormally high protein concentration may introduce significant error in test results.

ATOMIC ABSORPTION SPECTROPHOTOMETER

Atomic absorption spectrophotometry (AAS) is an analytical technique used to analyse metals at very low concentrations, typically in the ranges of parts per million (PPM) or parts per billion (PPB).

In this technique, a liquid sample containing dissolved analyte is aspirated into a thin, wide AA flame or is introduced into a small carbon furnace which is heated to a very high temperature. AAS has been used for clinical analysis of metals in biological fluids and tissues such as whole blood, plasma, urine, saliva, brain tissue, liver, hair, muscle tissue and semen. Analysis of metal content of water and drugs can also be done by this technique.

Principle

AAS measures absorption of radiation by free atoms. The total amount of absorption depends on the number of free atoms present in the sample and the degree to which the free atoms absorb the radiation. In order to analyze a sample for its atomic constituents, it has to be atomized. Atoms are freed from other types of atoms by high temperature of AA flame produced from either oxy-acetylene or nitrous oxide. Other types of AAS use electrothermal (graphite tube) atomizers. After the sample is broken down to different types of atoms, the amount of a specific atom is measured.

The atoms should then be irradiated by optical radiation, and the radiation source could be an element-specific line radiation source or a continuum radiation source. The radiation then passes through a monochromator in order to separate the element-specific radiation from any other radiation emitted by the radiation source, which is finally measured by a detector.

Components of AAS

Essential components of AAS include:

1. Sharp line radiation source (usually a hollow-cathode lamp)
2. A solution nebuliser and burner or furnace
3. Monochromator
4. Photomultiplier
5. Detector system

Interferences

Two common types of interferences are faced in AAS. These are spectral and chemical. **Spectral interferences** result from the overlap of absorption lines by molecular species. It can be corrected by matrix matching of sample and standards. **Chemical interference** arises due to stable compound formation and ionisation, both of which decrease amounts of free atoms in sample vapour.

Applications of AAS

Over 60 elements can be detected by AAS in, almost, any matrix, like body fluids, polluted water, foodstuff, soft drinks and alcoholic beverages. Besides, analysis of metallurgical and geochemical samples can also be done. Metals can also be measured in soil, crude oil, petroleum products and plastic, etc.

Advantages of AAS

1. Solutions, slurries and solid samples can be tested.
2. Atomisation is very efficient.
3. This technique is highly sensitive.
4. Smaller quantities of sample (5–50 mL, approx.) is needed.

Disadvantages of AAS

1. It is a highly expensive technique.
2. Requires high level of operator skill.
3. Low sample throughput.
4. Low precision.
5. Sample must be in solution or at least volatile.
6. Individual source lamps are required for each element.

CHEMILUMINESCENCE IMMUNOASSAY ANALYZERS

Chemiluminescence immunoassay (CLIA) is one of the immunoassay techniques where label or indicator of the analytical reaction is a luminescent reaction. In spectrophotometry, luminescence is better than absorbance, as the former is an absolute measure while the latter is a relative one.

Principle

It is based on the fact that light emission occurs from the excited singlet state, when the electron returns to the ground state. Since, chemiluminescent labels are used, the chemiluminescent molecules emit light when they are excited by chemical energy.

Commonly used chemiluminescent labels are **isoluminol** and **acridinium esters**. Oxidation of isoluminol by hydrogen peroxide produces a light emission at 425 nm in presence of catalyst while oxidation of acridinium esters by alkaline hydrogen peroxide, in presence of detergent, produces a flash of light at 429 nm.

Procedure

In CLIA for a hormone, antibody for the hormone is coated on microtiter wells. Then patient's serum with the hormone and hormone conjugated with horseradish peroxidase are added to the microtiter wells. During the incubation time, hormone in the patient's sample competes with the labeled hormone for binding with antibody on the wells. After the incubation, excess unbound hormone is washed. Thereafter, chemiluminescent substrate is added and the relative light units (RLU, intensity of emitted light) is read. RLU is directly proportional to the amount of enzyme present and inversely proportional to the amount of patient's hormone concentration.

Applications

CLIA has been used for:

1. Thyroid function markers
2. Reproductive hormones, like testosterone, estrogen and progesterone, prolactin, and β-HCG
3. Tumor markers, including AFP, CA-125, PSA
4. Vitamins, like folic acid and B_{12}
5. Anemia profile, such as ferritin and transferrin
6. Cardiac markers, e.g. troponin I and others.
7. Infectious disease markers
8. Autoantibodies

Advantages of CLIA

1. Wide dynamic range
2. High signal intensity
3. Absence of interfering emissions, so more sensitive.
4. High stability of reagents and their conjugates, and low consumption of reagents
5. Long incubation time is not required.
6. Addition of stopping reagent is not required

Disadvantages of CLIA

1. Limited antigen detection
2. High cost
3. Limited test panel
4. Closed analytical systems

FUTURE TRENDS IN AUTOMATION

Laboratory automation is an ongoing process and will continue to evolve. **Integrated systems** which can handle routine chemistry, immunochemistry, hematology and serology will be the next generation instruments. System integration with miniaturization with more technologically advanced computer(s)/power will persist to accommodate more portable analyzers, for more precise testing.

Future **automated analyzers will have artificial intelligence** whereby the computer will **think** or **make decisions**, if sufficiently programmed with infinite scenarios of data (spectral mapping or multiple wavelengths monitoring with high resolution photometers and polychromators will become standards).

Important Questions

1. Describe total laboratory automation in a clinical biochemistry laboratory.
2. What is a clinical chemistry autoanalyzer? Describe various types of clinical chemistry analyzers.
3. Differentiate between:
   i. Modular and integrated system of autoanalyzers
   ii. Close and open system autoanalyzers
   iii. Centrifugal and random access autoanalyzers
4. Write notes on:
   i. Discrete autoanalyzers
   ii. Continuous-flow autoanalyzers
   iii. Automation in clinical chemistry laboratory
   iv. Atomic absorption spectrophotometry
   v. Chemiluminescence immunoassay

Chapter

42

Organization of a Clinical Chemistry Laboratory

Clinical biochemistry deals with the biochemistry laboratory applications to find out the cause of a disease. The chemical constituents of various body fluids, such as blood (serum or plasma), urine, CSF and other body fluids are analyzed in a clinical biochemistry laboratory. These determinations are useful in diagnosing various clinical conditions such as diabetes mellitus, jaundice, gout, hyperlipidemia, pancreatitis, rickets, etc. Furthermore, biochemistry tests are carried out for many organ disorders, such as liver, stomach, heart, kidneys, brain, endocrine disorders and to know the status of acid–base balance of the body. The clinical biochemistry tests, in relation to the various clinical conditions can:

1. Reveal the cause of the disease
2. Screen easy diagnosis
3. Suggest effective treatment
4. Assist in monitoring progress of a pathological condition
5. Help in assessing response to therapy

When a patient approaches the laboratory with a requisition form, requesting tests, the lab technologist, with the help of other laboratory staff carries out the following duties:

- Instructing the patient
- Collecting the specimen
- Preserving the specimen
- Analyzing the specimen
- Preparing the reports
- Dispatching of the reports

The structure and function of a clinical laboratory varies according to the level of health care facility. Peripheral laboratories carry out simple tests, like urine analysis and hemoglobin estimation, whereas higher centers are equipped with sophisticated technology and trained manpower to carry out complex investigations.

Following requirements of clinical biochemistry laboratory are highly specific:

- Glassware and plasticware
- Reagents and diagnostic kits
- Equipment and instruments
- Automation
- Total quality management (TQM)

A STANDARDIZED CLINICAL CHEMISTRY LABORATORY SET UP

Application of good laboratory practice is an essential element of a quality assurance program. To begin with, it is necessary to have a standardized laboratory set up. Some essential factors for a standardized laboratory are given below.

Laboratory Premises

- Adequate working space is necessary.
- Premises should be clean, uncluttered and free from obstructions.
- Provision of uniform and slip resistant floors and stairs.
- Good condition of benches, furniture and fittings.
- Bench surfaces should be resistant to solvents and corrosive chemicals.
- Walls, ceilings and floors should be smooth, easily cleanable, impermeable to liquids and resistant to the chemicals and disinfectants that are normally used in the laboratory.
- Adequate circulatory spaces and corridors for the movement.
- Each laboratory room should have at least one wash basin.

Heating, Ventilation and Lighting

- Comfortable working temperature
- Exposed to sunlight

- Adequate ventilation
- Adequate lighting at work benches.

Services

- Each laboratory room be provided with enough sinks, water, electricity and gas outlets for safe working.
- Adequate inspection and maintenance programs for fuses, lights, cables, pipes, etc.

Fire Prevention Facilities

- Presence of a fire alarm system.
- Fire detection system in good working order and regularly tested.
- All exits marked by properly illuminated signs leading to an open space.
- Corridors aisles and circulation areas should be clear and unobstructed for movement of staff and fire-fighting equipment.
- All fire-fighting equipment and apparatus should be easily identified by an appropriate color code.
- All portable fire extinguishers should be fully charged, kept in working order and placed in designated places at all times.
- Every laboratory room should be equipped with appropriate extinguishers for emergency use.
- Sufficient mechanical ventilation to remove vapors of flammable liquids and gases before they reach a hazardous concentration.
- "No smoking" signs.

Electrical Installation

- All new electrical installations and all replacements, modifications and repairs should be made and maintained in accordance with electrical safety code.
- The interior wiring should have a grounded (earthed) conductor (i.e. a three-wire system).
- All electrical appliances should have testing laboratory approval.
- Each electric socket outlet should be used for only one appliance.
- All the electrical appliances should have testing laboratory approval.

Storage Facilities

- Storage facilities, shelves, etc. should be arranged properly so that stores are secure against sliding, collapse, falls or spillage.
- Storage facilities should be kept free from accumulation of unwanted materials and objects that present hazards from tripping, fire, explosion and harbourage of pests.
- Separate place should be reserved for the storage of flammable chemicals. This storage area should be ventilated with appropriate fire extinguishers. These areas should be adequately labeled with "no smoking signs" and fire hazard signs (*see* Chapter 7).

Sanitation and Staff Facilities

- Clean and adequate toilet (WC) and washing facilities should be provided for both male and female staff.
- Hot and cold water, soap and towels should be provided to the staff members.
- Provision should be made for a room for lunch and separate changing rooms for male and female staff members.
- Arrangement should be made for collection and disposal of rubbish.

Compressed and Liquefied Gases

- Compressed gas cylinders and their high pressure and reduction valves should be regularly inspected for safety.
- When gas cylinders are not in use or are being transported, the protection caps should be placed appropriately.
- Gas cylinders should be kept away from sources of heat.

Health and Safety of Staff

- First-aid boxes should be provided at strategic places.
- Laboratory workers and nonlaboratory workers should be instructed on the potential hazards of the laboratory.
- Prominent notices should be placed about the location of first aids and telephone numbers of emergency services.
- An immunization program relevant to the laboratory work should be implemented.
- The two persons rule should be applied whereby no individual ever works alone in the laboratory.
- There should be a periodic arthropoid and rodent control programs.
- A good quality and dependable water supply is essential. There should be no cross connections between sources of laboratory and drinking water supplies. Laboratory workers should have easy access to safe drinking water.

Chemicals and Radioactive Substances

- All chemicals should be correctly labeled with names and warnings.
- Chemical hazard warning charts should be displayed prominently.
- Staff should be trained to deal with spillage.
- Bottle carriers should be used appropriately.
- Proper record should be maintained of stocks and of radioactive substances.

Laboratory Equipment

- All equipment should be certified and safe for use.
- Procedures should be available for decontaminating equipment prior to maintenance.
- Biological safety cabinets and fume cupboards should be regularly tested and serviced.
- Appropriate plasticware should be used instead of glassware to avoid contact and reuse of broken, cracked and chipped glassware.
- Appropriate disinfectants should be used correctly.

Disposals

An identification and separation system for contaminated materials can be established as follows:

- Noncontaminated waste can be disposed off with general waste,
- Sharps, such as hypodermic needles, broken glass, knives and scalpels, and
- Contaminated material should be disposed separately (*see* Chapter 17).

Disinfection

Chlorine (sodium hypochlorite) is a universal disinfectant which is active against all microorganisms. Normally, it is available as sodium hypochlorite. It is a strong oxidizing agent and is corrosive to metal.

A general all-purpose laboratory disinfectant solution of sodium hypochlorite should have a concentration of 1 g/liter (1000 ppm) as available chlorine. In the presence of large amount of organic substances and for dealing with blood spillage a strong solution of sodium hypochlorite such as 5 g/liter (5000 ppm), as available chlorine, is recommended. Sodium hypochlorite solution sold for laboratory use contains 50 g/liter (50000 ppm) as available chlorine and, therefore, it should be diluted to 1:50 or 1:10, to obtain final concentration of 1 g/liter and 5 g/liter, respectively.

MANAGEMENT OF A CLINICAL CHEMISTRY LABORATORY

The objective of a clinical laboratory is to assist the clinicians with reliable reports of tests, which is a vital component of excellent healthcare delivery system. The clinical laboratory management requires expertise in medical, scientific and technical areas. The proper management of a laboratory is based on the integrated role of various personnel towards a common goal of achieving quality reports. The **laboratory director** directs and manages the activities of a laboratory. He decides upon the time and amount of supplies needed for the uninterrupted functioning of the laboratory. A systematic procurement system has to be developed. For this, the addresses of various manufacturers must be procured. The best product selection within the available funds can be done in consultation with several other clinical laboratories. In this context, he has to take the approval from the higher authorities of the institute. The head of the laboratory should also be conversant with the accreditation standards and governmental regulations.

Quality manager looks after the quality control of the laboratory assays. Both (internal quality control) IQC and (external quality control) EQC should be stringently followed for the accurate results by the laboratory. The external quality control coordinator also plays an important role in procuring the EQC samples from the specified sources and reports the results to the quality manager. **Technical manager** looks after the proper functioning, calibration and maintenance of various pieces of equipment periodically for their correct performance. A constant check on the expiry of chemicals and reagents should also be maintained. Record of consumables and the working of collection area of samples are also the responsibilities of technical manager.

Senior technicians look after the proper sample receiving along with their processing, analysis and reporting with the help of other lab technicians. Qualified persons should be recruited who should be given their proper job description. Duty allotment schedule should be prepared. Opportunities should be carefully provided for betterment of skills by means of seminars, workshops, etc. It is very important to maintain a good rapport between the person-in-charge and working staff (Fig. 42.1).

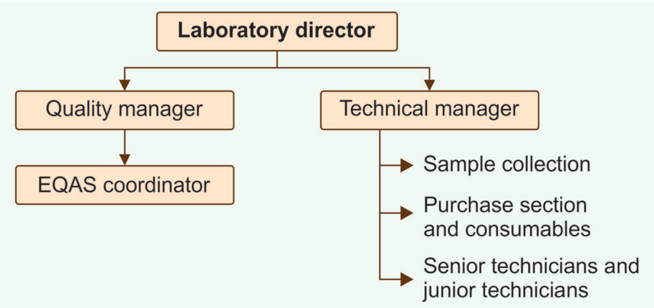


Fig. 42.1: Laboratory management set up (EQAS: External quality assurance service)

RECORD-KEEPING IN A CLINICAL CHEMISTRY LABORATORY

Record is basically a document that furnishes objective evidence of activities performed and of results achieved. The gold standard of record-keeping is based on the judicious use of physical permanent notebooks/registers. However, advances in technology has resulted in large amounts of electronic records making it virtually impossible to maintain a full set of records in physical notebooks. Electronic laboratory notebook systems aim to meet the stringency for keeping records electronically. Good record-keeping is necessary for the following reasons:

1. It provides a basic reference which the individual and others can refer to at a later date.
2. For data analysis, publication, collaboration, peer review and research activities.
3. It can defend against false allegation of research misconduct.

Instructions for Recording Data in the Laboratory Notebook

1. Lab notebook should be bound and with numbered pages. All entries should be made in legible and orderly manner using permanent ink.
2. If an error is made, cross it out and make the correction. Cancellations or insertions should be initialled, dated and explained, if possible.
3. Each day's work should be started on a separate page with lines drawn diagonally across the unused portion of the previous page. It is extremely important that each page shows the date of entry.
4. Each page should be signed and dated by the individual who makes the entry and does the work.
5. Notebook should be kept in the laboratory cabinet or office and protected from damage as they are legal evidence. The loss of a research notebook has to be reported.
6. The laboratory notebooks are the property of the organization and must be retained by the laboratory. The data of the patient's result should be preserved for at least one year beyond the award period, as required by NABL guidelines or in accordance with the institution's record retention policy.

Thorough documentation is a requirement for a good job performance. Efforts are useless to everyone, if one fails to document their work.

Important Questions

1. Describe the set up of a standardized clinical chemistry laboratory.
2. Write notes on:
   - i. Clinical chemistry laboratory management
   - ii. Record-keeping in a clinical chemistry laboratory

Chapter
43

Pancreatic Function Tests

The pancreas serves two functions which are carried out by different groups of cells within the organ. These two groups of cells are designated as the endocrine and the exocrine portions of the pancreas.

THE ENDOCRINE PANCREAS

The endocrine pancreas consists of group of cells known as the **islet of Langerhans**, which are embedded in the exocrine portion of the gland. Islets are composed of four major cell types, each of which synthesize and secrete a different hormone. These include glucagon producing alpha cells, insulin producing beta cells, somatostatin producing delta cells and a pancreatic polypeptide producing F cells.

THE EXOCRINE PANCREAS

The exocrine secretory cells are called acinar cells arranged in sac-like clusters called acini. These cells secrete a small volume of juice containing the digestive enzymes of the pancreas. The acini are connected by small intercalated ducts to larger ducts that converge into one duct which delivers the exocrine secretion of the pancreas into the duodenum. The epithelial cells lining the intercalated ducts secrete a relatively large volume of juice with a high concentration of the alkaline salt sodium bicarbonate.

Biochemical Functions of the Exocrine Pancreas

Exocrine functions of the pancreas, as described above, include the production and secretion of pancreatic juice which is rich in several enzymes and bicarbonate.

Total volume of pancreatic juice secreted in a normal human is 500–800 mL/24 hours. It is strongly alkaline with a pH of about 8.0. Its alkalinity is due to the presence of bicarbonate (HCO_3^-) since pancreatic juice contains nearly 70–100 mEq/L of HCO_3^-. By neutralizing acid that arrives in the duodenum from the stomach, bicarbonate performs two functions:

1. It protects the delicate lining of the upper small intestine from damage by acid.
2. It ensures optimal pH for activity of the pancreatic digestive enzymes.

The pancreas also secretes several digestive enzymes (Table 43.1).

Table 43.1 Digestive enzymes of the pancreatic juice

Enzymes	Substrates	Digestion products
Trypsin	Proteins and peptides	Smaller peptides
Chymotrypsin	Proteins and peptides	Smaller peptides
Carboxypeptidases A and B	Proteins and peptides	Smaller peptides and amino acids
Amylase	Starch	Oligosaccharides, maltose
Lipase	Triacylglycerols	Monoacylglycerols and free fatty acids
Phospholipase A_2	Lecithin	Lysolecithin and free fatty acid
Cholesterol esterase	Cholesterol esters	Cholesterol and free fatty acids
RNase	RNA	Ribonucleotides
DNase	DNA	Deoxyribonucleotides

Regulation of Exocrine Pancreatic Secretion

Secretion of the pancreatic juice is controlled by two mechanisms, i.e. by **hormonal control** and **nervous control** (via vagus nerve).

Two hormones, secretin and cholecystokinin, which are released from the duodenal mucosa during the intestinal phase of digestion regulate bicarbonate and enzyme secretion by the pancreas.

- ***Secretin*** is released into the blood in response to HCl. After its release, the hormone stimulates the intercalated duct cells of the pancreas to secrete bicarbonate-rich juice. The secretion, in turn, neutralizes the acid that causes secretin to be released and so reduces the stimulus for additional secretin release until more acid enters the duodenum from the stomach.
- ***Cholecystokinin*** is released from duodenal mucosa in response to protein and lipid digestion products. The hormone stimulates the pancreatic acinar cells to secrete digestive enzymes. As a result, more digestion products are produced which further stimulate the pancreas to secrete more enzymes. The process continues as long as food remains in the small intestine and is to be digested (Fig. 43.1).

  Vagus stimulation also helps in the secretion of enzymes.

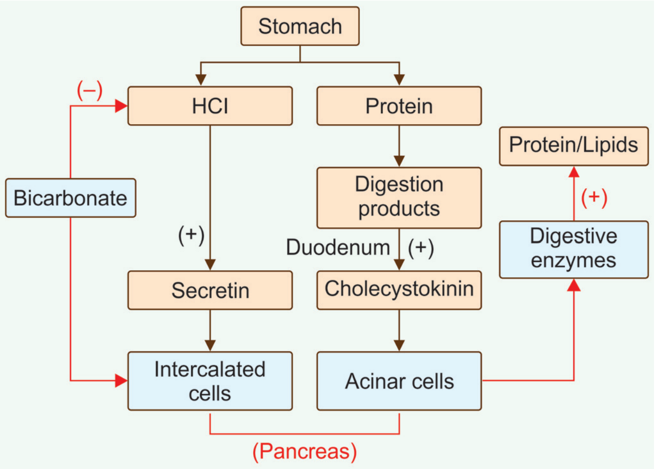


Fig. 43.1: Regulation of exocrine pancreatic secretion

BIOCHEMICAL TESTS TO ASSESS EXOCRINE FUNCTIONS OF THE PANCREAS

Volume and Bicarbonate Concentration of the Pancreatic Juice

The exocrine secretory capacity of the pancreas is assessed by intubating the duodenum and subjecting the pancreas to stimulation with a test meal, secretin or pancreozymin. After the duodenal contents are aspirated, the patient is given secretin (one unit/kg body weight) intravenously and the aspirate is examined for volume, bicarbonate content and amylase activity.

In pancreatitis, there is usually a reduction in the volume of pancreatic juice as well as its bicarbonate output. In the milder form of pancreatitis, volume of the pancreatic juice and its bicarbonate contents are fairly normal, but enzyme activities, particularly, the activity of amylase is reduced. Patients with chronic pancreatitis are unable to secrete juice of high bicarbonate content (<90 mEq/L). Carcinoma of the head of pancreas obstructs the overall volume flow.

Enzymes in Serum/Urine

Serum and Urinary Amylase

Serum and urine amylase activities are increased in acute pancreatitis. In early stages of the disease, determination of serum amylase is of great diagnostic importance. Rise starts within 2–12 hours of the onset of pain and usually returns to normal within 3–5 days. There is also an increase in urinary amylase.

Serum Lipase

Serum lipase activity rises slower than that of amylase, within 4–8 hours and normalizes by 7–14 days. Thus, it remains elevated for a longer duration than serum amylase. Further, serum lipase, as compared to serum amylase, is elevated more often in patients with pancreatic carcinoma.

Serum Elastase

Elastase I is a pancreatic proteolytic enzyme that hydrolyzes scleroproteins, a basic ingredient of the connective tissue. It is produced in the acinar portion of the pancreas and appears in the exocrine pancreatic secretion as zymogen (proelastase). This, in turn, is activated by trypsin. Serum elastase I is of significance in the later stages of acute pancreatitis or in patients with relapse or cystic complications of chronic pancreatitis. Elastase I remains abnormally elevated longer than amylase.

Important Questions

1. Describe the set up of a standardized clinical chemistry laboratory.
2. Write notes on:
   i. Clinical chemistry laboratory management
   ii. Record-keeping in a clinical chemistry laboratory

Chapter

44

Liver Function Tests

The liver is a multifunctional organ that is involved in a number of excretory, synthetic and metabolic functions (Table 44.1).

Table 44.1 Important biochemical functions of the liver

Metabolism of nutrients
• Carbohydrates • Proteins • Fats
Synthesis of proteins
• Albumin • α- and β-globulins • Clotting factors • Transport proteins
Metabolism and excretion of exogenous substances
• Alcohol • Drugs • Toxins
Metabolism and excretion of endogenous substances
• Bilirubin • Hormones
Storage of vitamins
• Vitamin A • Vitamin B_{12}
Minerals
• Iron

1. The liver has a major influence on the flow of nutrients to the rest of the body. It controls the release of absorbed material into the systemic circulation and has a central role in carbohydrate, protein and fat metabolism.
2. The liver is an important site for the storage of glycogen and for gluconeogenesis.
3. Most proteins in plasma including albumin, α and β-globulins, clotting factors and transport proteins are synthesized by the liver.
4. This is also the site for urea synthesis.
5. Fatty acids and phospholipids are also taken up from circulation by the liver and are metabolized there.
6. Cholesterol is also synthesized and esterified in the liver.
7. Liver is also a site for the detoxification of exogenous compounds (such as alcohol, drugs and toxins) as well as endogenous materials (such as bilirubin and hormones) which are excreted via the kidney or the biliary system.
8. Bile salts are synthesized in the liver.
9. Liver stores various substances, such as vitamins and minerals, and releases them as and when required.
10. Liver is also one of the biggest reticuloendothelial organs and has important immune functions.

COMMONLY USED BIOCHEMICAL TESTS FOR THE ASSESSMENT OF THE FUNCTIONS OF LIVER

Various liver function tests that are useful in the assessment of a liver disease include the estimation of serum proteins, serum bilirubin, various enzymes and prothrombin time (Table 44.2).

Table 44.2 Commonly used biochemical tests for the assessment of liver functions in serum

Total proteins
Albumin **Bilirubin** **Prothrombin time** **Enzymes** • GPT and GOT • Alkaline phosphatase • γ-Glutamyltranspeptidase (γ-GT) • 5′-Nucleotidase • Glutamate dehydrogenase

Serum Bilirubin

Bilirubin is an orange-yellow pigment, derived from the breakdown of red blood cells in the liver, spleen and bone marrow (*see* Chapter 33).

Its daily production, in men, averages from 250 to 300 mg. Approximately, 85% of this is derived from the heme moiety of hemoglobin which is released from the erythrocytes that are destroyed in the reticuloendothelial cells while rest of it is formed from catabolism of other heme containing proteins, such as myoglobin, cytochromes and other heme containing enzymes.

Bilirubin, normally present in the blood, is bound to albumin and is transported to the liver.

Hepatocytes trap bilirubin by means of a specific binding protein called **ligandin. In the hepatocytes, bilirubin** gets **conjugated with UDP-glucuronate,** which is derived from the oxidation of UDP-glucose. **This reaction is catalyzed by UDP-glucuronyl-transferase.**

In the normal bile, bilirubin diglucuronide is the major form of excreted bilirubin with only a small amount of the bilirubin monoglucuronide. As bilirubin diglucuronide is much more water-soluble than free bilirubin, transferase, thus, facilitates the excretion of bilirubin, via bile duct, into the intestine.

As **bilirubin diglucuronide** is poorly absorbed by the intestinal mucosa, glucuronide residues are **released in the terminal ileum and large intestine, by intestinal β-glucuronidases and by the enzymes produced by anaerobic bacteria. It is reduced to** colorless linear tetrapyrroles, called stercobilinogen and urobilinogen. These compounds are collectively referred to as urobilinogens.

A large portion of **urobilinogens are excreted in the feces**.

Majority of these re-circulating pigments are taken up by the liver and re-excreted in the bile. Some of the urobilinogens (only up to 2%) are also reabsorbed passively from the colon and return to the liver via the portal venous blood. This is referred to as **enterohepatic circulation** (Fig. 44.1).

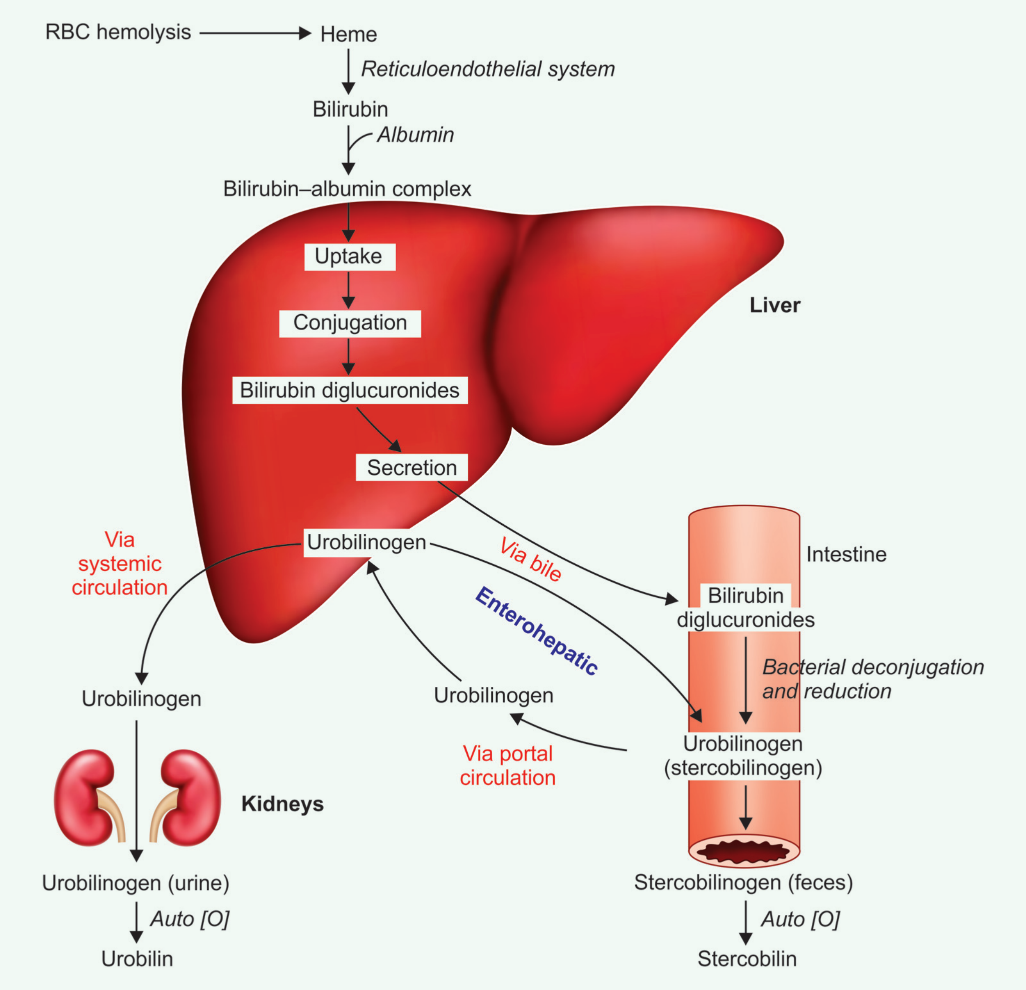

RBC hemolysis
Heme
Reticuloendothelial system
Bilirubin
Albumin
Bilirubin–albumin complex
Uptake
Conjugation
Bilirubin diglucuronides
Secretion
Liver
Urobilinogen
Via bile
Via systemic circulation
Enterohepatic
Intestine
Bilirubin diglucuronides
Bacterial deconjugation and reduction
Urobilinogen
Urobilinogen
Via portal circulation
Urobilinogen (stercobilinogen)
Kidneys
Urobilinogen (urine)
Auto [O]
Urobilin
Stercobilinogen (feces)
Auto [O]
Stercobilin

Fig. 44.1: Enterohepatic circulation

Most of the reabsorbed urobilinogen is taken up by the liver and is re-excreted in the bile. **A small portion of urobilinogen (2–5%)**, however, escapes hepatic tissue, **reaches the peripheral circulation and is excreted in the urine.**

Serum, thus, contains two different forms of bilirubin which are referred to as:

1. **Unconjugated** (lipid-soluble) form that is bound to albumin and is transported from the reticuloendothelial system to the liver.
2. **Conjugated** (water-soluble) form which is regurgitated from the liver into the plasma.

Normal value for the sum of the unconjugated and conjugated forms is 0.1–1.0 mg/100 mL of serum or plasma.

Normally, almost all the bilirubin in plasma is unconjugated.

Disorders of bilirubin metabolism lead to **hyperbilirubinemia** where serial measurement of bilirubin is helpful in knowing the severity of a liver disease.

Bilirubin fractionation is also helpful in differential diagnosis of **jaundice.**

JAUNDICE

Jaundice is a physical sign characterized by yellow appearance of the patient and is a most characteristic clinical manifestation of hyperbilirubinemia. It results from the deposition of bilirubin (bile pigment) in the skin, mucous membrane and sclera of the patient. It is apparent clinically, when serum bilirubin level is more than 2 mg/dL. If serum bilirubin is below 2 mg/dL, it is called **latent jaundice** (subclinical jaundice) since it is not detectable at this stage, clinically.

Classification and Evaluation

There are three types of jaundice, referred to as pre-hepatic, hepatic and post-hepatic jaundice.

Pre-hepatic Jaundice

Pre-hepatic jaundice is also called hemolytic jaundice. It is characterized by the excessive presence of unconjugated bilirubin. It may be a result of increased production of unconjugated bilirubin such as in hemolysis, decreased uptake of unconjugated bilirubin across the hepatocyte membrane as in Gilbert syndrome or decreased biotransformation such as in neonatal jaundice, Crigler-Najjar syndrome, etc.

Hemolysis

In disorders associated with hemolysis (hemolytic anemia), rate of bilirubin production is increased which may exceed the amount that cannot be removed by the liver.

Gilbert syndrome

This is a heterogeneous group of disorders, inherited as autosomal recessive trait. Several defects include the deficiency of bilirubin glucuronyltransferase, a defect in hepatic uptake of bilirubin or a decrease in red cell survival.

Neonatal jaundice

Neonatal jaundice is also called as physiological jaundice of the newborn. Every infant exhibits some transient unconjugated hyperbilirubinemia (about 5 mg/dL) between the second and the fifth day of life, as at this stage hepatic glucuronyltransferase activity is not fully developed. Activity of the enzyme increases within 2 weeks after birth when serum bilirubin returns to normal.

Neonatal jaundice is more pronounced in a premature infant.

Unconjugated bilirubin levels above 18 mg/dL lead to deposition of bilirubin in the lipid-rich basal ganglia. This is referred to as **kernicterus.**

Crigler-Najjar syndrome

This disorder is known to exist in two forms:

- **Type I:** It is clinically more severe form of the disease. It is due to complete absence of glucuronyltransferase.
- **Type II:** It has moderate clinical findings and is due to partial deficiency of the enzyme.

In both the conditions unconjugated bilirubin is significantly raised.

Biochemical evaluation of three types of jaundice can be done, as shown in Table 44.3.

Table 44.3 Differential diagnosis of different types of jaundice based on various biochemical tests

Biochemical parameter	Type of jaundice		
	Pre-hepatic	Hepatic	Post-hepatic
Serum bilirubin	↑↑	↑↑	↑↑
Type of bilirubin	Unconjugated	Both	Conjugated
Urine urobilinogen	↑↑	N/↑	↓ / -
Urine bile pigments	--	--	↑
Stercobilinogen	↑	↓	↓/ -
SGPT/SGOT (mainly SGPT)	N	↑↑↑	↑
Serum alkaline phosphatase	N	↑	↑↑↑
Serum 5′-nucleotidase	N	↑	↑↑↑
Prothrombin time (PT)	N	↑	↑

Hepatic Jaundice

Hepatic jaundice is due to a primary disease. Plasma, usually, shows elevated levels of both conjugated as well as unconjugated bilirubin, e.g. in viral hepatitis, liver cirrhosis, etc. There is also an increase in SGOT and SGPT levels.

Elevated levels of conjugated as well as unconjugated bilirubin along with the dark-brown pigmentation in the liver cells is also observed in a benign, autosomal recessive condition known as **Dubin-Johnson syndrome.**

Inhalation, ingestion or parenteral administration of the several pharmacological and chemical agents such as carbon tetrachloride, acetaminophen, isoniazid or chlorpromazine, which result in liver injury, also lead to hepatic jaundice.

Post-hepatic Jaundice

Post-hepatic jaundice is also referred to as **obstructive jaundice** or post-hepatic cholestasis. It is caused by obstruction of the bile duct, may be due to gallstones, carcinoma of the head of pancreas or carcinoma of the bile duct. Serum of such a patient shows excessive amount of conjugated bilirubin. It is also characterized by the presence of the excessive amount of bilirubin (bile pigments) in the urine.

Urinary Urobilinogen

Normally, only about 1–4 mg of urobilinogen is excreted in the urine, in 24 hours.

Increased excretion of urobilinogen may occur when hepatocellular function is impaired. Urobilinogen excretion in the urine may also increase if there is an excess of urobilinogen in the gastrointestinal tract, which exceeds the capacity of the liver, e.g. in hemolysis, viral hepatitis, cirrhosis, etc.

In contrast, when biliary excretion of bilirubin is impaired, e.g. in cholestasis, urinary excretion of urobilinogen is decreased due to limited delivery of bilirubin to the gut and low rate of production of urobilinogen. This, in turn, results in clay-colored or chalky-white stool in patients with cholestasis jaundice.

Plasma Proteins

As liver is the primary site of the synthesis of plasma proteins, a disturbance in protein synthesis occurs as a consequence of impaired hepatic function. Decreased synthesis of protein leads to a decrease in their plasma concentration. Pattern of alteration in plasma proteins, however, depends upon the type, severity and duration of the liver injury.

In a severe liver disease, hepatic synthesis of albumin is decreased. In chronic hepatocellular damage also, serum albumin concentration falls.

Serum protein electrophoresis, to determine the proportion of different serum proteins, is also helpful in the diagnosis of a liver disease, e.g. α_2- and β-globulins are increased in cirrhosis due to their increased production and decreased clearance. In cholestasis, there is a small increase in serum γ-globulin with a more increase in α- and β-globulins due, in part, to increased serum lipoproteins.

Serum Enzymes

Serum levels of several enzymes are elevated in liver diseases.

SGOT/SGPT

In viral hepatitis and other forms of a liver disease, which may be associated with hepatic necrosis, serum GPT and GOT levels are elevated even before clinical signs and symptoms of the disease appear. Levels of both the enzymes may reach values as high as 100 times the upper normal limit. Peak values occur between day seven and day twelve. Thereafter, their activities gradually decrease and reach toward normal by third to fifth weak of recovery.

Five to ten-fold increase in both the enzymes occur in patients with primary metastatic carcinoma of the liver. Although serum levels of both GPT and GOT are elevated in a liver disease, however, GPT levels are, comparatively, more elevated, as it is more liver specific.

Alkaline Phosphatase

Serum alkaline phosphatase levels are important in a hepatobiliary disease as well as in bone diseases that are associated with increased osteoblastic activity.

As biliary obstruction results in induction of the enzyme, rise is more marked in extrahepatic obstruction than in intrahepatic obstruction.

Glutamate Dehydrogenase

Glutamate dehydrogenase (GDH) is found in the liver, heart, muscle and kidney. It is present only in traces in normal serum but increased activity is observed in a liver disease with hepatocellular damage.

5′-Nucleotidase

5′-Nucleotidase (5′-NT) activity is increased by two to six folds, in some of the hepatobiliary diseases. It may be due to extrahepatic causes such as stone or tumor, obstructing the bile duct or in intrahepatic conditions, e.g. cholestasis that may be caused by chlorpromazine, malignant infiltration of the liver or biliary cirrhosis.

γ-Glutamyltransferase

γ-Glutamyltransferase is also referred to as γ-glutamyltranspeptidase or γ-GT. It is present in high concentration in the serum in intrahepatic or post-hepatic biliary obstruction (*see* Chapter 51).

Elevated levels of γ-GT are also observed in the sera of people who are heavy drinkers.

This enzyme has been shown to be of significance in the detection of an alcohol-induced liver disease.

PROFILE OF LIVER FUNCTION TESTS

A useful combination of tests, which can be done to determine whether a liver disease is present and to give some indication of the etiology, include **serum bilirubin, glutamate pyruvate transaminase (SGPT or alanine aminotransferase), alkaline phosphatase, γ-glutamyltranspeptidase (γ-GT) and albumin.** Examination of the **urine** for the amount of **urobilinogen** can also be done. Additional tests, which study some liver function tests, such as galactose tolerance test and the hippuric acid synthesis test can be done in doubtful cases.

In Jaundice Patients

In jaundice patients, **unconjugated hyperbilirubinemia** is usually due to hemolysis or Gilbert syndrome. In hemolysis, **bilirubin is absent from the urine** and **urinary urobilinogen** concentration **is increased**.

In conjugated hyperbilirubinemia (increase in both conjugated and unconjugated bilirubin) large **increase in SGPT** suggests hepatocellular damage, which may be caused by drugs, toxic agents (like alcohol), or infection (viral hepatitis).

On the other hand, marked **elevation in γ-GT and alkaline phosphatase** suggests **cholestasis** (obstruction).

Plasma albumin concentration provides a fairly good index of the **progress of a chronic liver disease.**

Normal values of the various parameters are shown in Table 44.4.

Table 44.4 Normal serum concentration of some of the liver function test parameters, which are done routinely

Parameters	Normal values
Plasma albumin	3.5–5.5 g/dL
SGOT activity	Up to 40 U/L
SGPT activity	Up to 56 U/L
Alkaline phosphatase activity	80–300 U/L
Total bilirubin	<0.8 mg/dL
γ-GT activity	5–35 U/L

Important Questions

1. Describe hepatic function tests. How will you use these tests for differential diagnosis of different types of jaundice?
2. Describe the role of bilirubin as a liver function test to differentiate between different types of jaundice.
3. Write notes on:
   - i. Jaundice
   - ii. Neonatal jaundice
   - iii. Biochemical functions of the liver.

Chapter

45

Gastric Function Tests

The stomach functions as a temporary storage organ for food. It secretes about 2–3 liters of gastric juice every day. Most of this juice is produced by exocrine glands located in the body and fundus of the stomach. These exocrine secretory glands contain three types of secretory cells, i.e. the chief cells, the parietal cells and the mucous cells.

The chief cells

These are located in the basal region of these glands and also in the glands of the antrum, and secrete **pepsinogen** (the inactive precursor of pepsin).

The parietal cells

These secrete **HCl**. In addition to converting pepsinogen to pepsin, HCl provides optimum pH for the activity of this enzyme. The parietal cells also secrete **intrinsic factor** necessary for the intestinal absorption of vitamin B_{12} which is required for formation of normal RBCs.

The mucous cells

These are located at the neck of these glands, in the glands of the antrum and scattered throughout the gastric epithelial lining. These cells secrete **alkaline mucous containing fluid** which coats the gastric wall and protects against various forms of damage to the gastric mucosa.

BIOCHEMICAL FUNCTIONS OF THE STOMACH

Secretion of HCl

The important function of the stomach is that parietal cells secrete an iso-osmotic solution of HCl with a hydrogen ion concentration of ≈150 mEq/L (pH 0.9–1.0). Both excitatory and inhibitory mechanisms regulate gastric HCl secretion. The gastric mucosa is rich in histamine, a potent stimulus of parietal cell secretion. During the cephalic phase, there is release of acetylcholine as well as gastrin, both of which cause the release of histamine in the vicinity of the parietal cells.

Secretion of Pepsin

Pepsinogen, the inactive precursor of pepsin, is synthesized by and stored in the chief cells as zymogen granules. Once pepsinogen enters the gastric lumen, HCl and pre-existing pepsin convert it to pepsin. Both acetylcholine and peptides stimulate secretion of HCl by the parietal cells and, thus, in turn, play an important role in the activation of pepsin as well as in the maintenance of the highly acidic environment required for its optimal activity.

EVALUATION OF GASTRIC FUNCTION TESTS

The content of the stomach after nearly 12 hours of fast is referred to as **gastric residue (residuum)**. Gastric juice consists of water (99.4%), hydrochloric acid, mucin and several enzymes such as pepsin, carbonic anhydrase and lipase.

- **Hydrochloric acid** is secreted by the parietal cells.
- **Pepsin** is secreted as pepsinogen, by the chief cells. HCl activates it to pepsin.
- **Rennin** is secreted in the fourth stomach of calf and young ruminants, and in infants. It curdles milk, by converting casein into para-casein which further reacts with calcium ions to form Ca-paracaseinate.
- **Gastric mucin** comprises mucoid, mucoprotease and glandulary mucoprotein. Mucoprotein is the main carrier of the intrinsic factor that is necessary for the absorption of vitamin B_{12} (which is referred to as the extrinsic factor).

Factors Affecting Secretion of Gastric Juice

The secretion of gastric juice is increased reflexly, in response to various stimulants:

1. Psychic stimuli such as thought, sight, smell and taste of food.
2. Mechanical stimuli, e.g. presence of food in the stomach.
3. Chemical substances like histamine, alcohol, etc.
4. Hormone gastrin secreted by gastric mucosa, in response to the food present in stomach.
5. Secretion of gastric juice may be inhibited by fat and HCl.

Chemical Composition of Gastric Juice

Hydrochloric Acid

Parietal cells secrete iso-osmotic solution of HCl, with a hydrogen ion concentration of about 150 mEq/L (pH near 0.9–1.0). It is practically free of ions other than H^+ and Cl^-. It is due to the reason that parietal cells contain high concentration of carbonic anhydrase.

HCl performs several functions

1. HCl of gastric juice initiates **conversion of pepsinogen to pepsin**.
2. It has some **physical action on proteins** and makes them more easily digestible.
3. It also **converts** the colloidal **ferric hydroxide**, which is found in the foodstuffs, **to ferric ions** that can be more readily reduced to ferrous ions by ascorbic acid, cysteine and sulfhydryl groups of proteins of the food.

Zollinger-Ellison Syndrome

It is due to a gastrin-secreting tumor of the endocrine pancreas. Excess gastrin overstimulates gastric acid secretion resulting in abdominal pain, mucosal ulceration and diarrhea. It is suspected in patients of acid-peptic disease who fail to respond to conventional treatment with H2 receptor blockers and proton pump inhibitors.

Gastric acidity may be decreased by various factors

- Variations in the rate of parietal secretion.
- Dilution by the secretions of other cells, particularly, mucus.
- Dilution and buffering by food.
- Dilution and buffering by the swallowed saliva.
- Regurgitation of the duodenal fluid and bile.

Pepsin

Pepsin is present in the chief cells, as the zymogen (pepsinogen). Pepsinogen is converted to the active enzyme (pepsin) by the acidity of gastric juice and also autocatalytically by pepsin itself.

Acetylcholine and peptides stimulate secretion of HCl by the parietal cells and, thus, play an important role in the activation of pepsin as well as maintenance of acidic pH, which is required for its optimal activity.

Pepsin content is high in patients having peptic ulcer. In pernicious anemia, cirrhosis of the liver and in various chronic gastric ailments pepsin content is low.

Other Acids

Besides HCl, gastric residue contains mucoproteins and organic acids such as lactic acid and butyric acid, etc., which contributes to the total acidity of the gastric juice. Total acid concentration in a normal state is usually between 10 and 50 mEq/L.

Stimulation of Gastric Secretion

Many compounds or processes such as test meal, alcohol, caffeine, gastrin, pentagastrin or insulin stimulate gastric secretion and, in turn, the secretion of HCl.

Test Meals

Stimulation of gastric secretion by administration of a test meal such as toast with water or tea (Ewald's meal) was practiced in the past. This test, however, is not used these days.

Alcohol

50–100 mL of 7% ethyl alcohol (3.5 mL ethanol in 50 mL water) may be administered through the syringe attached to the tube as a stimulant for HCl secretion.

Caffeine

Caffeine sodium benzoate, 500 mg in about 200 mL of water, may be administered into the stomach through a gastric tube as a stimulant of gastric secretion.

Histamine

Histamine is a powerful stimulant for the secretion of gastric juice. It causes secretion of an increased volume of highly acidic gastric juice with low pepsin content. Two types of histamine tests may be carried out.

In the ***'standard histamine test'***, subcutaneous injection of histamine (0.01 mg/kg body weight) is given and the gastric contents are removed. Absence of free HCl in gastric secretion after histamine indicates achylia gastrica.

A large dose of histamine (0.04 mg/kg body weight) is given for the ***'augmented histamine test'***. In pernicious anemia no free HCl is secreted but in other forms of achlorhydria some amount of free HCl may be secreted, after augmented histamine stimulation test is performed.

Antihistaminics

Sometimes a high dose of histamine may cause a severe reaction mediated through H1 receptors. Under such a condition, an injection of antihistaminic may be given, intramuscularly. This does not interfere in gastric stimulation because the gastric effects of histamine are mediated through H2 receptors.

Insulin

Intravenous injection of 0.1–0.2 U/kg body weight of soluble insulin may also be given for the stimulation of gastric secretion and gastric samples are withdrawn after every 15 min. This is called ***Hollander test***.

Insulin stimulation is helpful to ascertain the effectiveness of vagotomy in patients with duodenal ulcer. In these patients, concentration of free HCl may be as high as 1200 mEq/L. After successful vagotomy, since there is no response to insulin, gastric acidity remains at a low level of 15–20 mEq/L before as well as after insulin administration.

As insulin may cause hypoglycemia the test is not preferred.

Gastrin and Pentagastrin

Gastrin is the most powerful stimulus for gastric HCl secretion. Gastrin is administered in a single subcutaneous dose of 2 mg/kg body weight.

Pentagastrin, a synthetic pentapeptide with a physiologically active part of the gastrin molecule, is also a potent stimulus for gastric HCl secretion and is commonly used these days. The fasting content of the stomach is aspirated and its volume is measured. This is termed as the **basal acid output** (BAO). The secretions are collected at 15 min intervals for 1 hour. Pentagastrin is then given at a dose of 6 mg/kg body weight subcutaneously and post-stimulation secretions are again collected at 15 min intervals for the next one hour. This acid output is termed as the **maximal acid output** (MAO).

This test is useful in differentiating the diagnosis of achlorhydria and hyperchlorhydria. In normal persons, the BAO ranges from 0 to 10 mEq/hour. After stimulation with pentagastrin, MAO varies from 0 to 35 mEq/hour. A large volume of fasting juice indicates obstruction of gastric outlet. A very high BAO suggests that the patient has Zollinger-Ellison syndrome.

Collection of Gastric Juice

The content of the stomach after nearly 12 hours of fast is defined as gastric residue (residuum). It is obtained by aspiration through a nasogastric tube (**Ryle's tube**) which is a thin rubber tube having lead bead at the end and a perforation just above the bead. The patient comes after an overnight fast and this tube is introduced through one of the nostrils after proper lubrication and is made to reach the gastric antrum. The position is depicted by markings on the tube and also can be assessed radiologically by the position of the lead bead. The contents are then aspirated by a syringe and examined, physically and chemically.

Basal Acid Output

Fasting content of stomach is aspirated. Secretions are collected at 15 minutes intervals, up to 1 hour, pooled and volume is measured. This is termed as **basal acid output (BAO).**

Maximal Acid Output

After collection of the residuum, pentagastrin (6 mg/kg body weight) is given subcutaneously and post-stimulation secretions are again collected at 15-min intervals, up to next one hour. This acid output is termed as **maximal acid output (**MAO). It is useful in differentiating the diagnosis of achlorhydria and hyperchlorhydria.

Examination of the Gastric Residue

Physical Examination

Volume

Total volume of the resting gastric residue normally varies from 20 to 50 mL. An increase in the volume (>100 mL) may be due to hypersecretion, retention or regurgitation from duodenum and is considered abnormal. Various causes include pyloric obstruction, duodenal ulcer or Zollinger-Ellison syndrome.

Consistency

The resting gastric juice is fluid in consistency. It may be viscous in the presence of mucus. Food particles may be present in carcinoma of the stomach.

Color

The resting gastric content is usually colorless. It may be slightly yellow or green due to regurgitation of bile, or red/brown due to the presence of blood.

Chemical Examination

Bile

It may be found in gastric residuum as a result of intestinal obstruction or ileal stasis.

Mucus

Small amount of mucus is found normally. Increased amounts may be seen in gastritis and gastric carcinoma.

Blood

Accidental trauma to the gastric mucous membrane during the passage of Ryle's tube may result in traces of blood in the gastric juice. Increased amount of blood may be present due to gastric carcinoma, erosive gastritis or bleeding peptic ulcer.

Test for blood: It may be detected by benzidine test. Take 3 mL of saturated benzidine solution, 2 mL of gastric juice and 1 mL of hydrogen peroxide in a test tube. Appearance of deep blue color indicates the presence of blood.

Organic acids

Organic acids, mainly, present in the gastric juice, include lactic acid and butyric acid. They may be present in large amounts in conditions of hypoacidity, achlorhydria and carcinoma of the stomach. In the absence of free HCl, these organic acids are formed in increased amounts by bacterial fermentation of food, as food is retained in the stomach for longer period (usually more than 6 hours) at neutral or alkaline pH.

Test for lactic acid: Take 2 mL of gastric juice in a test tube and add few drops of 10% ferric chloride solution to it. Appearance of yellow color indicates the presence of lactic acid.

Estimation of Free and Total Acidity of Gastric Juice

Free Acidity

The main acid in the gastric juice is HCl which is a strong mineral acid. It accounts for free acidity. HCl is secreted by the parietal cells at a concentration of about 150 mEq/L with pH <1.0. As the parietal secretion gets mixed with other gastric constituents, the concentration of hydrogen ions usually reaches <40 mEq/L with a pH of 1.5–2.5.

Total Acidity

Total acidity in gastric residue includes hydrogen ions occurring as free HCl, mucoproteins, acid salts and organic acids, such as lactic and butyric acids. The total acid concentration in a normal state is usually 10–50 mEq/L.

Principle

Acids present in the gastric juice are titrated with a strong alkali. During titration with alkali (0.1 N NaOH), HCl is completely neutralized at about pH 3.5, whereas weak acids are neutralized in the alkaline range (at about pH 8.0). Thus, titration is done by using Topfer's reagent as indicator, which has two components: dimethyl yellow and phenolphthalein. Dimethyl yellow is used during titration for free acidity while phenolphthalein is used for titrating total acidity.

Methyl yellow has a pH range of 2.9 (red) to 4.0 (yellow). On the other hand, phenolphthalein works in the pH range of 8.2 (colorless) to 10.0 (pink).

Procedure

Fill 0.1 N NaOH solution in a burette. Take 10 mL gastric juice in a conical flask or China dish and add a drop of Topfer's reagent to it. If initially, on adding the indicator, yellow color is seen, no free acid is present. Titrate this sample only for total acids.

If initially red color appears, titrate it with 0.1 N NaOH till salmon pink color appears. At this stage, the initial red color becomes yellow. Note the volume of NaOH used. This gives the value of free acidity.

Again titrate with 0.1 N NaOH. Note the change in color from salmon pink to colorless, which further turns pink. Note down total NaOH used for titration. This gives the value for total acidity.

Calculation

$$\begin{array}{c}\text{Free acidity}\\ \text{(mEq/L)}\end{array} = \begin{array}{c}\text{ml of 0.1 N NaOH required to titrate}\\ \text{10 mL of gastric juice with Topfer's}\\ \text{reagent (dimethyl yellow; A)}\end{array} \times \frac{100}{10}\text{, or}$$

$$= A \times 10$$

$$\begin{array}{c}\text{Total acidity}\\ \text{(mEq/L)}\end{array} = \begin{array}{c}\text{ml of 0.1 N NaOH required to}\\ \text{titrate 10 mL of gastric juice}\\ \text{with phenolphthalein (B)}\end{array} \times \frac{100}{10}\text{, or}$$

$$= B \times 10$$

The result may also be expressed in terms of **clinical units.**

One clinical unit is defined as the **amount of 0.1 N NaOH required to neutralize** the amount of acids present in **100 mL of gastric juice.**

Reference Value

Concentration of **free acidity** in gastric residue in normal individuals varies from 0 to 40 mEq/L.

Total acidity is, generally, up to 10 mEq/L more than free acidity. Thus, total acidity in a normal state is usually 10–50 mEq/L.

Interpretation

Free acidity concentration above 50 mEq/L may be suggestive of **hyperacidity**. When free acidity is increased, the condition is called hyperchlorhydria. It occurs in duodenal and gastric ulcers, and in diseases of the gall bladder.

Complete absence of HCl is referred to as **achlorhydria**. It occurs in pernicious anemia. Absence of free HCl in gastric residue is considered abnormal, only, if the condition persists after maximal stimulation with pentagastrin. Complete absence of gastric secretion is referred to as achylia gastrica. This is observed in advanced cases of gastric carcinoma.

In the absence of HCl, bacterial fermentation of food is increased which leads to the formation of lactic acid. A small percentage of the healthy individuals (about 4% of the young ones and 25% at age 60 years) may have no free HCl in the fasting stomach.

PRACTICAL EXERCISES

1. Perform physical and chemical examination of gastric juice.
2. Determine free and total acidity of gastric juice.

Important Questions

1. Describe various biochemical functions of the stomach.
2. Describe various gastric function tests.
3. Write notes on:
   - i. Zollinger-Ellison syndrome
   - ii. Antihistaminics
   - iii. Stimulation of gastric secretion
   - iv. Free and total acidity of gastric juice
   - v. Major constituents of gastric juice along with their functions
   - vi. Basal acid output
   - vii. Maximal acid output

Chapter 46

Kidney Function Tests

Various metabolic processes occurring in the body produce a number of substances, many of which, such as CO_2, water, nitrogenous compounds and inorganic salts, are waste products. If not regularly removed from the body, these toxic metabolites may severely affect normal health. The kidneys excrete most of the water as well as organic and inorganic substances, whereas CO_2 is expired through lungs.

FUNCTIONS OF THE KIDNEY

Kidney performs several biochemical functions (Table 46.1).

Table 46.1 Important functions of the kidney

- Filtration of blood and preparation of ultrafiltrate fluid as urine
- Reabsorption of useful substances such as glucose, amino acids, electrolytes, etc.
- Homeostasis of the extracellular volume.
- Maintenance of the acid–base status, water and electrolytes
- Endocrine functions and activation of hormones such as synthesis of erythropoietin, activation of vitamin D, etc.

EXCRETION OF WASTE PRODUCTS

Urine is an ultrafiltrate of plasma. It is formed in the kidney, a paired organ, which is meant for filtration, concentration and reabsorption of various constituents. Each kidney has nearly one million basic filtration units called nephrons.

Nephron

A **nephron** consists of the **glomerulus** and the **tubule.**

The **glomerulus** is a **network of arteriolar afferent and efferent capillaries** and is **surrounded by** a double-walled epithelial sac called **Bowman's capsule.** Bowman's capsule leads to the **tubule,** which is **divided into proximal convoluted tubule, loop of Henle and distal convoluted tubule.**

Loop of Henle consists of the descending limb, a thin segment and the ascending limb. Proximal convoluted tubule has its **opening into the descending limb of the loop of Henle,** which **continues into the thin segment, from where ascending limb arises. It continues into the distal convoluted tubule, which finally opens into the collecting duct** (Fig. 46.1).

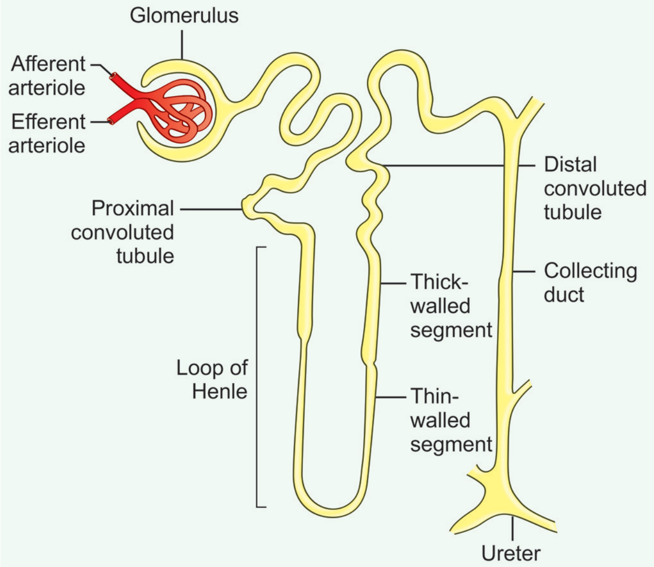


Fig. 46.1: Nephron

Filtration of Blood

Filtration is carried out in the nephron through glomeruli. As blood passes through the glomeruli, noncolloidal substances (usually of low molecular weight), filter through capillaries, with the retention of protein (not normally found in the urine).

Total amount of the filtrate formed per minute, by all the nephrons of both the kidneys, is known as **glomerular filtration rate** (GFR). Under normal physiological conditions, **GFR is 125 mL/min.**

Reabsorption of Useful Substances

About 150–200 L of the ultrafiltrate passes through the glomeruli in 24 hours. Reabsorption of water and solutes in various regions of the tubule reduces its volumes to nearly 500–1800 mL per day, as urine.

In the proximal tubule nearly 60–80% of the ultrafiltrate is absorbed in an obligatory fashion, along with **Na^+, Cl^-, HCO_3^-, Ca^{2+}, HPO_4^{2-}** and other ions. **Glucose** is reabsorbed almost completely, predominantly in the proximal tubule by a Na^+-dependent active transport process. **Uric acid** is also reabsorbed in the proximal tubule. When blood level of creatinine increases above normal, creatinine is secreted in this region.

In the loop of Henle, Cl^- and more of the Na^+ but without water are reabsorbed, resulting in diluted urine. Water reabsorption is then regulated in the distal tubule and collecting duct by antidiuretic hormone (ADH or vasopressin).

In the distal tubule secretion is the prominent activity as organic ions; K^+ and H^+ are transported from the blood in the efferent arteriole into the tubular fluid. This region also secretes H^+ and reabsorbs Na^+ and HCO_3^-.

At the same time, certain solutes are added to the fluid by an excretory function of the tubule. These include hippuric acid and other organic waste products.

Substances, which are reabsorbed by the renal tubules, are divided into two groups, according to their threshold value:

High Threshold Substances

These compounds are vital for life and body cannot afford to loose them. Such substances are completely reabsorbed by the renal tubule, e.g. glucose, amino acids, etc.

Glucose is reabsorbed in the tubular epithelial cells.

Sodium, potassium, amino acids and other substances are reabsorbed, apparently by specific transport mechanisms.

Transfer of **carbon dioxide and bicarbonate** is controlled by carbonic anhydrase.

Low Threshold Substances

These compounds are not required by the body and are reabsorbed to a very small extent only, e.g. urea, creatinine, etc.

Urine thus carries off

1. Water and salts in such amounts so as to maintain normal equilibrium between extracellular and intracellular fluids
2. Acids or bases, to maintain a normal acid–base balance
3. Waste products
4. Toxic and detoxified substances
5. Other substances, those are present in the blood in excessive amounts.

Regulation of Water and Electrolytes

Regulatory functions of the kidney have a major role in homeostasis, by maintaining constant optimal chemical composition of the blood as well as interstitial and intracellular fluids.

Regulation of Water

The kidney plays an important part in regulating water balance. If excessive amount of water (fluid) is ingested, kidney excretes the same. On the other hand, if intake is low, kidney excretes more concentrated urine.

Water homeostasis is intrinsically linked to renal urea handling. Only 40–50% of the filtered load of urea is reabsorbed.

Most of the glomerular filtrate is reabsorbed through tubule cells.

About 90% of water is reabsorbed as a result of the difference in pressure. The remaining 10% of water is reabsorbed actively, by tubular cells, as a result of the action of ADH.

In the absence of ADH little water and urea are reabsorbed, and a large amount of water is excreted. On the other hand, in the presence of ADH only 1.0–1.5 L of the filtrate is excreted as urine.

Regulation of ADH excretion is thus of vital importance to fluid homeostasis.

Regulation of Electrolytes

The kidney plays an important role in the maintenance of electrolyte balance. Salts are either excreted or conserved by the kidney by tubular reabsorption, depending upon dietary intake and physiological need.

Plasma electrolytes pass through the glomerulus but some potassium leaves the blood by way of tubules, by active secretion.

Sodium and Chloride

Sodium and chloride ions predominate in the glomerular filtrate, as in plasma. As the glomerular filtrate flows through tubule, these electrolytes are absorbed into the blood.

Reabsorption of Na^+ is required for the reabsorption of water, Cl^-, glucose, HCO_3^-, urea and amino acids.

The proximal tubule is highly permeable to Na^+. Approximately 80% of the Na^+ entering tubular cells do so in exchange for H^+ secretion, which, in turn, leads to the entry of both Cl^- and HCO_3^- into the cells.

Sodium transport is regulated by many factors

- Protein kinase-dependent phosphorylation
- Aldosterone
- Na^+, K^+-ATPase
- Na^+-H^+ exchanger
- H^+-ATPase

Calcium and Phosphorus

Approximately 98% of filtered Ca^{2+} is reabsorbed. Out of which 65–75% in the proximal tubule, 20–25% in the thick ascending limb of the loop of Henle, 10% in the distal tubule and a small amount in the collecting duct.

A passive process that is linked to active Na^+ reabsorption predominantly absorbs calcium. However, in the distal tubule Ca^{2+} reabsorption is an active process catalyzed by Ca^{2+}-ATPase.

Reabsorption of Ca^{2+} is increased by the action of PTH and calcitonin.

Normally, less than 20% of the filtered load of phosphorus is excreted in the urine. Its excretion is directly related to the filtered load when plasma phosphorus concentration is above normal.

Reabsorption of phosphorus, though dependent on Na^+ reabsorption, is regulated by:

- Protein kinase C-dependent phosphorylation
- PTH
- Active vitamin D
- Calcitonin
- Dopamine and serotonin

Maintenance of Acid–Base Balance

Oxidation of various metabolites in the living organism results in the formation of a variety of acids as well as bases. For example, protons are generated from ionization of various substances such as organic acids (produced during metabolism of glucose, fatty acids and amino acids), uric acid (from purines), sulfates (from sulfur-containing amino acids) and carbonic acid (from CO_2).

Similarly, bases arise during catabolism of various metabolites such as ammonia (from amino acids), bicarbonate, phosphates, acetate and citrates.

All these substances enter blood plasma and other extracellular fluids for disposal. In spite of the entry of these acids and bases, pH of the blood does not alter much and remains within the normal limit of 7.35–7.45. This is due to the presence of various defense mechanisms, which include:

1. Buffer systems of the blood, tissue fluids and cells
2. Excretion or retention of CO_2 by lungs
3. Excretion of an acid or alkaline urine
4. Formation and excretion of ammonia as well as organic acids.

Regulation of Acid–Base Balance

Four reactions are mainly involved in the regulation of acid–base balance by the kidneys.

1. Exchange of H^+ for Na^+

Na^+ that is present in the lumen of the renal tubule, i.e. in the ultrafiltrate of plasma, is exchanged for H^+, which is formed in the tubular epithelial cells from CO_2 and H_2O. Na^+ is transported from the epithelial cells into the blood plasma for recycling. This transport of Na^+ is mediated by sodium pump.

In the presence of carbonic anhydrase, CO_2 and H_2O form carbonic acid, which is dissociated into H^+ and HCO_3^-. HCO_3^- diffuses from the epithelial cells into the blood plasma as the accompanying ion to Na^+, and thus, HCO_3^- is conserved.

2. Reabsorption of HCO_3^- from the Tubular Urine

The H^+ that is exchanged into the tubular urine reacts with HCO_3^- in the plasma ultrafiltrate and forms H_2CO_3. This is dissociated to CO_2 and H_2O from where CO_2 diffuses back into the epithelial cells.

3. Formation and Excretion of NH_3

Glutamine is extracted from the renal blood plasma of the peritubular capillaries and is deaminated by glutaminase in the tubular epithelial cells, to release NH_3 and glutamate.

NH_3 so formed is diffused into the tubular urine and binds with H^+ to form NH_4^+.

4. Excretion of H^+

The H^+ ions that are exchanged for Na^+ bind to HPO_4^{2-} to form $H_2PO_4^-$ (titratable acid). Both $H_2PO_4^-$ and NH_4^+ are then excreted in the urine. The accompanying ions are predominantly, Na^+ and Cl^-, respectively.

Kidney, thus, participates in the regulation of acid–base balance, primarily, by maintaining the HCO_3^- concentration in the plasma.

In the event of acidosis, excretion of both $H_2PO_4^-$ and NH_4^+ are increased in the urine while the reverse occurs in alkalosis, until acid–base balance is restored.

Synthesis and Activation of Hormones

Kidney synthesizes some hormones such as erythropoietin, renin and prostaglandins. It is also the site for the activation of vitamin D.

COMMONLY USED BIOCHEMICAL TESTS FOR THE EVALUATION OF KIDNEY FUNCTIONS

Commonly used biochemical tests for the assessment of the functions of kidney are shown in Table 46.2.

Table 46.2 Commonly used biochemical tests for the assessment of the kidney functions

Routine laboratory tests	Tests for glomerular functions	Tests for tubular functions
Urine • Volume • pH • Normal constituents • Abnormal constituents **Blood** • Urea • Creatinine • pH, gases and electrolytes	**Renal clearance test** • Inulin clearance • Urea clearance • Creatinine clearance **Renal plasma flow**	• Urine concentration test • Urine dilution test • Urine acidification test

EXAMINATION OF URINE

Physical Examination

Volume

Volume of the urine varies from person to person and time to time. Average volume of urine, excreted by a normal individual, over a period of 24 hours, is **1000–1500 mL**. It is influenced by diet, particularly, ingestion of fluids. Strenuous physical exercise, hot weather and fever diminish urine output.

Certain pathological conditions alter urine output. If 24 hours volume is increased, the condition is called **polyuria**, such as seen in diabetes and early renal failure.

When urine output is decreased, it is termed as **oliguria** such as seen in acute renal failure, dehydration (diarrhea and vomiting), edema and urinary obstruction.

In acute renal failure or shock, urine is usually not formed. This condition is called **anuria.**

Color

Normal urine is usually pale, straw-yellow colored, due to the presence of urochrome, uroerythrin and urobilin.

Change in color may be due to some pathology, secondary to the intake of some drug, or a particular type of food.

In fever it is usually dark in color, if there is blood in the urine (due to hemorrhage in the kidney or the urinary tract), urine becomes red in color. In jaundice urine may be dark-yellow or green due to the oxidation of bilirubin to biliverdin.

General Appearance and Odor

Freshly excreted urine is normally clear and transparent, as all the substances are present in the soluble form. On standing, it becomes turbid due to precipitation of phosphates.

Pathological urine may be turbid due to the presence of fat globules or pus cells.

Odor of the urine is usually aromatic, due to the presence of volatile acids. On standing, urine may develop pungent odor due to formation of ammonia from urea because of its decomposition by bacteria.

In acidosis and diabetes mellitus, urine has a peculiar sweet smell due to the presence of ketone bodies.

Specific Gravity

Specific gravity of urine normally varies between 1.010–1.030. Volume has a great influence on specific gravity of urine. Low values (up to 1.003) are observed after large water intake, on diuretic therapy or excretion of large amount of ketone bodies. High values (up to 1.040) are seen in dehydration or due to the presence of large amount of glucose.

Specific gravity of urine is measured by means of hydrometer, known as urinometer. Mark on the urinometer, in contact with surface of urine, indicates specific gravity of the sample.

pH (Reaction)

Under normal circumstances urine is slightly acidic in reaction, with a **mean pH of about 6.0 (ranging from 4.8 to 8.0)**

The pH of urine varies widely depending upon the nature of the food taken. High protein diet leads to acidic urine while alkaline urine is excreted after a vegetarian diet, particularly after large intake of vegetables and fruits.

Following meal, pH of the urine becomes alkaline due to the production and secretion of HCl by the stomach.

Alkaline urine is seen in patients with systemic alkalosis since body attempts to conserve H^+ ions and remove fixed bases. Strongly acidic urine is seen in patients with systemic acidosis, e.g. diabetes mellitus, due to the excretion of large amount of ketone bodies.

An increase in pH, greater than 8.0, indicates urinary tract infection by an organism that splits urea to ammonia.

The pH of urine is routinely checked with the help of pH paper. Its reaction can be tested with the help of litmus paper.

Chemical Examination

Normal Constituents

Major constituents of normal urine are shown in Table 46.3.

Table 46.3 Normal constituents of urine

Constituent	Daily excretion (g/24 hours)
Water	1000–1500 mL
Total solids	50–60
Organic substances	30–35
• Urea	25–30
• Uric acid	0.5–1.0
• Creatinine	1.0–2.0
• Free amino acids (as amino acid N)	0.15–0.20
Inorganic substances	20–25
• Chloride (as NaCl)	10–15
• Total sulfates (as sulfur)	0.7–1.0
• Total phosphates (as inorganic phosphorus)	0.8–1.1
• Ammonia	0.5–0.7

Normally, 50–60 g of solids are excreted in the urine over a period of 24 hours. About half of the total solids are urea. It comprises nearly 80–90% of total organic substances. Other organic constituents of normal urine include creatinine, uric acid, hippuric acid and ethereal sulfates.

Major inorganic substance excreted is chloride (as sodium chloride). It constitutes about 25% of the total solids excreted in 24 hours. Other inorganic constituents normally excreted in the urine include ammonia, phosphates and sulfates.

In certain pathological conditions these constituents may be excreted in abnormal amounts.

Organic Substances

- **Urea:** Urea is a principal end product of protein catabolism in mammals. An adult man excretes 25–30 g of urea under normal conditions, in 24 hours.

  Urea excretion is increased on high protein diet or in protein catabolic states such as fever.

  On low protein diet urea excretion is reduced. Decrease in urea excretion is also observed in disorders which are associated with impaired liver function, such as cirrhosis as well as in a kidney disease, such as nephritis.
- **Uric acid:** Uric acid is derived from nucleic acids by the oxidation of purine bases. It is found in urine to the extent of 0.5 to 1.0 g/24 hours. Its daily excretion, however, varies, depending upon the diet and several other factors. On a purine-free diet, uric acid output may be reduced up to 0.1 g/24 hours. On a high purine diet, uric acid excretion may be increased up to 2.0 g/24 hours.

  Pathologically, uric acid excretion is increased in gout and leukemia.
- **Creatinine:** Creatinine (a waste product) is anhydride of creatine. Creatinine is present in considerable amount in muscle, in the form of creatine phosphate. Very little of creatine is found in the urine.

  On the other hand, about 1.0 to 1.8 g of creatinine is excreted by an adult in 24 hours. Excretion of creatinine is related to the amount of body creatine, i.e. muscle mass. Daily excretion of creatinine is, therefore, fairly constant and is used to check the accuracy of 24 hours urine collection.

  Creatinine content of urine is increased in conditions associated with increased muscular activity.

  A decrease in creatinine excretion may be observed in disorders, which are associated with muscular weakness as well as in a kidney disease.
- **Amino acids:** Due to high threshold, normally only small amount of free amino acids are found in the urine. Their daily excretion (as amino acid nitrogen) is about 150–200 mg. Infants excrete more amino acids as compared to adults. During pregnancy excretion of amino acids is increased.

  Amino aciduria may be prominent in certain liver diseases and after poisoning by chloroform or carbon tetrachloride.

  In certain metabolic diseases increased excretion of some of the amino acids may be observed, such as arginine, cystine, lysine and ornithine in cystinuria.

Inorganic Substances

- **Chloride:** Chloride is a major inorganic constituent of the normal urine. Its excretion is dependent upon chloride content of the food. On an average daily output of chloride (expressed as sodium chloride) varies between 10 and 15 g. Fasting as well as excessive perspiration decreases its urinary output.

Pathologically, its excretion may be decreased in chronic nephritis, fever, burn, diarrhea and vomiting.

- **Phosphates:** Phosphates are present in the urine in various forms, e.g. alkaline phosphates, such as sodium, potassium and ammonium phosphates, which are derived from the ingested inorganic phosphate or earthy phosphates such as phosphates of calcium and magnesium. These are insoluble phosphates and get precipitated in alkaline urine.

  Excretion of phosphorus is dependent upon diet but on an average nearly 1.0 g of phosphates (as inorganic phosphorus) is excreted in 24 hours.

  Urinary phosphorus is increased in acidosis and certain bone diseases, e.g. rickets and osteomalacia.

  Phosphorus excretion is reduced in pregnancy, renal and infectious diseases as well as in hyperparathyroidism.
- **Sulfates:** Sulfates arise in the urine from the oxidation of sulfur, which is obtained from the sulfur-containing amino acids. Only a small amount of the ingested sulfur is excreted.

  Sulfates are present in the urine in various forms, e.g. **inorganic sulfates** such as sodium, calcium and magnesium sulfates; **ethereal sulfates** which are mostly the detoxification products, and **neutral sulfates,** e.g. cystine, taurine, etc.

  Under normal conditions about 1.0 g of sulfates (as sulfur) are excreted in the urine daily.
- **Ammonia:** Tubular cells of the kidney form ammonia. Precursor for ammonia is the amide nitrogen of glutamine. A decrease in blood pH speeds up ammonia production.

  Normally, about 0.5–0.7 g of ammonia is excreted in 24 hours. Although very little of it is present in a fresh sample of the urine, its level increases on standing, due to bacterial decomposition of urea.

  Ammonia excretion is decreased in a kidney disease while its excretion is increased in acidosis such as in diabetic ketoacidosis.

Abnormal Constituents

In the process of filtration of plasma through glomeruli, large molecular weight substances such as proteins are retained while small molecular weight substances, which are useful for the body, are reabsorbed. Normally, their amounts are so low that they are not detectable, by the routine laboratory tests.

Under certain pathological conditions, when either their metabolism is disturbed or a tubular damage occurs, large quantities of these substances are excreted in the urine. Hence, these substances are called **abnormal constituents of urine.** These include sugar (glucose), ketone bodies, protein (albumin), blood, bile salts and bile pigments (Table 46.4).

Table 46.4 Abnormal constituents of urine

Constituent	Conditions in which excreted
Sugar (glucose)	Diabetes mellitus, renal glycosuria
Ketone bodies	Prolonged starvation, lowcarbohydrate high-fat diet, diabetic ketoacidosis
Protein (albumin)	Nephrotic syndrome, glomerulonephritis
Blood	Hematuria, hemoglobinuria
Bile salts/pigments	Different types of jaundice

Sugar

Excretion of, readily detectable amount of sugar in the urine is known as mellituria or glycosuria, whereas excretion of glucose is referred to as glucosuria.

When blood glucose concentration exceeds 180 mg/ 100 mL such as in diabetes mellitus, re-absorptive capacity of the tubule is reduced and glucose appears in the urine. Glucose may also be found in the urine in hyperthyroidism, hyperpituitarism, hyperadrenalism or I cell disease of the pancreas.

Glucosuria may also be observed in a proximal tubular disease that impairs re-absorptive mechanism. This is known as **renal glucosuria.** In this condition, glucose utilization as well as blood glucose level may be normal.

Besides glucose, other sugars that may be excreted in the urine include lactose, galactose, fructose or pentoses.

Lactosuria may be observed in lactating women.

Fructose

It may be present in the urine after ingestion of large quantity of honey. Essential fructosuria is an inborn error of metabolism due to the deficiency of the enzyme fructokinase.

Pentoses

These may be found after ingestion of large quantities of fruits like plums and cherries. Pentosuria, referred to as L-xylulosuria, is an inborn error of metabolism due to the deficiency of xylitol dehydrogenase.

Galactosuria may be observed in infants suffering from congenital galactosemia. Galactosuria may also be seen in infants as well as adults with a liver disease.

Ketone Bodies

Ketone bodies (acetoacetate, β-hydroxybutyrate and acetone) are formed in considerable amounts when fat is metabolized, excessively. Their accumulation in the blood is called ketonemia while their excretion in the urine is referred to as ketonuria. Ketonemia and ketonuria occur in a condition, which is referred to as ketosis.

Ketosis may occur during prolonged fasting or carbohydrate deprivation. Pathologically, ketone bodies appear in the urine in diabetic ketoacidosis.

Proteins

Normal urine contains only traces of protein, which cannot be detected by routine procedures. When readily detectable amount of protein is seen in urine, this condition is called proteinuria. Since it is mainly the albumin that is detected abnormally, hence, proteinuria may also be referred to as albuminuria.

Proteinuria or albuminuria may be physiological (benign albuminuria) or pathological (renal albuminuria).

- **Physiological proteinuria** is usually transitory. There is no evidence of kidney damage but proteinuria may be observed after severe exercise, high protein diet, pregnancy or postural effect (when an individual assumes an upright position for a long period). The later is called **orthostatic** or **postural proteinuria.**
- **Pathological proteinuria** may be classified as:
  - Pre-renal proteinuria
  - Renal proteinuria
  - Post-renal proteinuria
- ***Pre-renal proteinuria:*** Pre-renal proteinuria may be observed in conditions that are primarily not related to the kidney, e.g. a cardiac disease, by affecting the circulation of the kidney, may lead to proteinuria. Besides, fever, convulsions, anemia and liver diseases may also result in proteinuria.
- ***Renal proteinuria:*** Renal proteinuria may be observed due to inflammation of the kidney (nephritis) or kidney damage (nephrosis). Proteinuria due to a glomerular disease can lead to nephrotic syndrome where large amount of protein is excreted.

  Acute tubular necrosis and pyelonephritis also lead to proteinuria, which is generally mild.
- ***Post-renal proteinuria:*** Post-renal proteinuria may occur due to some inflammatory, degenerative or traumatic lesions of the pelvis of the kidney, ureter, bladder, prostate or urethra.

Blood

Either intact red blood cells or hemoglobin may be present in the urine.

Presence of red blood cells in the urine is referred to as **hematuria.** It can be confirmed by the presence of red blood cells by microscopic examination of the urine. RBCs may be seen in acute glomerulonephritis or trauma to the urinary tract.

Hemoglobinuria may be observed due to intravascular hemolysis. This, in turn, results in the liberation of the hemoglobin which is filtered in the glomerulus and appears in the urine. Hemoglobinuria may occur under various pathological conditions such as mismatched transfusion of blood, hemolytic jaundice, typhoid or malaria.

Bile

Bile is produced in the liver. It is secreted through biliary tract and enters duodenum. It is alkaline in reaction and bitter in taste. Its color varies from yellowish-brown to dark green. Important constituents of the bile are bile salts, bile pigments, lipids particularly phospholipids and cholesterol, proteins like mucin, urea and certain inorganic salts such as sodium chloride and bicarbonate.

- **Bile salts** are the sodium and potassium salts of glycocholic and taurocholic acids. Cholic acid has the basic ring structure of cyclopentanoperhydrophenanthrene, like cholesterol. In glycocholic acid, glycine is conjugated with cholic acid through a peptide linkage. Similarly, in taurocholic acid, taurine is conjugated with cholic acid. Bile salts are essential for the digestion of fat. Since fat is insoluble in water, it is not digested in the absence of bile salts. Bile salts emulsify fat by lowering surface tension of water and help to bring fat in intimate contact with the lipase.
- **Bile pigments** are the degradation products of hemoglobin. When hemolysis of red blood cells takes place, hemoglobin is dissociated into heme and globin. Globin is hydrolyzed to amino acids by the proteolytic enzymes, while heme looses its iron, which is reutilized for heme synthesis. The tetrapyrrole ring (protoporphyrin) is broken down to biliverdin which is further oxidized to bilirubin. Bilirubin is predominant in human bile. When it enters the intestine, further changes take place due to bacterial action and a number of pigments are formed. Some of these are reabsorbed into blood through the intestine, modified and excreted through urine.

Urobilinogen

Urobilinogen is an important constituent of the freshly voided normal urine. Following its oxidative degradation, it disappears quickly. Increased amount of urobilinogen may be seen in the urine of patients with hemolytic jaundice.

QUANTITATIVE ESTIMATION OF SOME OF THE CONSTITUENTS OF BLOOD

Blood Urea

Urea is a major nitrogenous metabolic product of protein catabolism in man, accounting for more than 75% of the nonprotein nitrogen which is eventually excreted. Urea is freely filtered by the glomeruli. In a normal kidney, 40–70% urea moves passively out of the renal tubule into the interstitium.

Blood urea level, in a normal individual, may vary between 15 and 45 mg/dL. In patients with untreated chronic renal failure blood urea level may be increased to 100–150 mg/dL.

Measurement of blood urea is widely used as a test of renal function. However, a number of nonrenal factors influence circulating urea concentration. For example, urea production and consequently blood urea concentration are increased on a high protein diet, in protein catabolic states, gastrointestinal hemorrhage and treatment with cortisol in some cases of chronic liver disease and with decreased perfusion of the kidney. Blood urea also depends upon the state of hydration of the patient.

Estimation of plasma urea concentration, however, is a less valuable test of renal function than plasma creatinine concentration. This is due to the reason that nearly 50% of the urea, filtered at the glomerulus, is passively reabsorbed through the tubules and this fraction increases if urine flow rate increases, such as in dehydration. Plasma urea concentration is also more affected by diet than plasma creatinine.

Low blood urea concentration may be observed in several situations, such as reduced synthesis, e.g. on a low protein diet or in an acute liver disease. Blood urea level may also fall as a result of water retention.

High blood urea concentration may be due to several causes, which may be subdivided into pre-renal, renal and post-renal:

- ***Pre-renal causes*** of increase in blood urea concentration include increased production by the liver (such as on a high protein diet), hemorrhage in the upper gastrointestinal tract, or in protein catabolic states (e.g. due to trauma, major surgery, or starvation). It may also be due to impaired renal perfusion (e.g. extracellular fluid loss, cardiac failure, hypoproteinemia, etc.).
- ***Renal causes*** of increase in blood urea concentration include acute or chronic renal failure with reduction in glomerular filtration.
- ***Post-renal causes*** include obstruction to urine flow, such as stone, benign prostatic hypertrophy, malignant stricture, or obstruction.

Serum Creatinine

Creatinine is a waste product of creatine (*see* Chapter 31). Its daily excretion is related to muscle mass and does not vary greatly from day to day. Hence, its level in the blood remains fairly constant. Creatinine is freely filtered at the glomerulus. Although it is not reabsorbed to any great extent by renal tubules but a small amount of it is secreted. Its secretion is increased with the increasing level of plasma creatinine.

Normal serum creatinine concentration varies between 0.8 and 1.5 mg/dL.

Meal, containing meat, may increase serum creatinine concentration. Certain drugs, e.g. salicylates, cimetidine, etc., also increase serum creatinine level.

Serum creatinine values are lower in children than in adults, in women than in men, and during pregnancy. Determination of serum creatinine gives a useful indication of the degree of renal failure.

An increase in serum creatinine, due to fall in GFR is observed when:

1. There is decreased renal perfusion, e.g. reduced blood pressure, fluid deprivation or renal arterial stenosis.
2. There is a loss of functional nephrons, e.g. in acute and chronic glomerulonephritis.
3. Pressure is increased on the tubular side of the nephron, e.g. in urinary tract obstruction due to prostate enlargement.

Serum creatinine concentration is a more precise measurement than creatinine clearance and is usually sufficient for following the progress of patients with renal disease. Because:

1. Plasma creatinine concentration normally remains fairly constant throughout adult life, whereas creatinine clearance declines with advancing age.
2. Plasma creatinine concentration correlates well with GFR as does creatinine clearance in patients with renal disease.
3. Measurements of plasma creatinine concentration are as effective in detecting early renal disease as creatinine clearance.
4. Sequential plasma creatinine concentration measurements enable the progress of renal disease to be followed with better precision than creatinine clearance.

Reduced plasma creatinine concentration is found in subjects with a small total muscle mass. Thus, a lower value is found in children. Values are, on average, normally lower in women than in men. Abnormally low values may be found in wasting diseases (reduced muscle bulk) and starvation, and in patients on steroid therapy, due to their protein catabolic effect. Plasma creatinine concentration is also usually low in pregnancy.

Increased plasma creatinine concentration may be observed in several nonrenal situations. For example:

1. High meat intake can cause temporary rise in plasma creatinine level.
2. Transient small increase in plasma creatinine level may occur after vigorous exercise.
3. Some drugs, e.g. salicylates, cimetidine, etc., reduce tubular secretion of creatinine hence, exhibit elevated plasma creatinine concentration.
4. High concentration of certain compounds, such as acetoacetate or cephalosporin antibiotic exhibit analytical interference and result in its overestimation.

An increased plasma creatinine concentration indicates a fall in GFR. Renal causes of increased plasma creatinine level include:

1. A disease, in which there is impaired renal perfusion, e.g. reduced blood pressure, fluid depletion, renal artery stenosis, etc.
2. Diseases in which there is loss of functioning nephrons, e.g. glomerulonephritis.
3. Urinary tract obstruction due to prostatic enlargement.

Blood pH, Gases and Electrolytes

Diminished capacity of the kidney to excrete H^+ results in metabolic acidosis, the severity of which may be assessed by measuring arterial H^+, HCO_3^- and pCO_2 concentrations.

Estimation of plasma sodium, potassium, calcium and phosphorus are also of value in certain kidney conditions, e.g. chronic renal failure or metabolic acidosis accompanied by hyponatremia, hyperkalemia, hypocalcemia and hyperphosphatemia.

Uric Acid

An increase in serum uric acid is found to correspond roughly with the increase in urea. With increasing renal failure, serum uric acid is markedly increased. However, estimation of uric acid is of little use in renal disease; it may be due to the reason that several other factors also influence uric acid level.

EVALUATION OF KIDNEY FUNCTION TESTS

Kidney functions can be evaluated with respect to either glomerular functions or tubular functions.

TUBULAR FUNCTION TESTS

These include urine concentration, dilution and acidification tests.

Urine Concentration Test

Urine concentration test is done by measuring osmolality (number of solute particles per kg water) of the specimen of urine after water deprivation or administration of vasopressin (ADH).

Restriction of water results in maximum stimulation of ADH secretion, in a normal subject. Since ADH acts on collecting tubules and promotes reabsorption of water, it results in concentrated urine.

For the test, the subject is not given water or any other fluid for 8 hours. Thereafter, osmolality of the urine is measured which should be 800 mOsm/kg. Specific gravity of this sample should be 1.022. If the urine sample has less osmolality or specific gravity, it indicates impaired renal concentrating power of the kidney either due to a tubular defect or decreased ADH secretion.

Urine Dilution Test

Urine dilution test is done to assess ability of the kidney to eliminate excessive amount of water.

After an overnight fast, the subject is asked to empty the bladder and given 1000 mL of water to drink within 20 minutes. In a normal subject 750 mL of it is excreted within 4 hours. During this period, urine samples are collected after every one-hour. Specific gravity of one of the specimen should be 1.003 while its osmolality should be less than 100 mOsm/kg. If renal function is impaired, quantity of water eliminated in 4 hours will be reduced. Moreover, specific gravity of the urine will be 1.010 or higher.

The test is not advisible in patients with adrenal insufficiency.

Urine Acidification Test

Ability of the kidney to produce acidic urine is also an important function of the tubule.

For this test, person is asked to empty the bladder and given ammonium chloride (0.1 g/kg body weight, in a gelatin capsule). Thereafter, urine samples are collected each hourly, for 8 hours. The pH of the urine during this period should be nearly 4.6–5.0.

In patient with renal tubular acidosis, pH of the urine does not fall below 5.3.

GLOMERULAR FUNCTION TESTS

Glomerular function tests include renal clearance of various endogenous as well as exogenous substances such as urea, creatinine or inulin and renal plasma flow.

Renal Plasma Flow

By measuring excretion rate of a substance, which undergo both, glomerular filtration as well as tubular secretion, e.g. p-aminohippuric acid, we can measure renal plasma flow (PAH).

As determined with PAH clearance, renal plasma flow for normal adult male is 700 ± 130 mL/min.

Renal Clearance

Renal clearance of an endogenous or exogenous substance, such as urea, creatinine, inulin, etc. is an expression of glomerular function of the kidney.

CLEARANCE TEST FOR RENAL FUNCTION

Clearance is defined as the volume of plasma that is cleared-off a particular substance by the two kidneys in one minute. It is calculated by the following mathematical relationship:

$$C = \frac{U \times V}{P}$$

where:

C = Clearance of the substance (mL/min)

U = Concentration of the substance (mg/dL) in urine

V = Rate of flow of urine (mL/min)

P = Concentration of the substance (mg/dL) in plasma, blood or serum.

Marker for clearance test

Suitable marker of renal clearance should meet the following criteria, i.e. it should:

1. Be freely filterable at the glomerulus.
2. Not be reabsorbed by the tubules.
3. Not be secreted by the tubules.
4. Be present at a stable plasma concentration.

For this purpose, either an **exogenous substance**, such as inulin, or an **endogenous substance** like urea or creatinine is used.

Inulin Clearance

Inulin is a polymer of fructose. It is filtered by the glomeruli, but is neither reabsorbed nor secreted by the tubule. Inulin clearance, therefore, is nearly equal to glomerular filtration rate (GFR).

Normal value for GFR, as per inulin clearance, found in adults, is about 125 mL/min.

Although inulin meets most of the criteria for measuring GFR, it is not suitable for routine use, because it is an exogenous substance and has to be administered intravenously.

Urea Clearance

As discussed in Chapter 31, urea is an end product of protein catabolism. Its concentration varies depending on dietary protein intake. Further, after filtration from the glomeruli, it is both, secreted from as well as reabsorbed by the tubules, thus, its excretion is affected by the rate of flow of urine.

As both, blood urea as well as urine urea concentrations are variable; accordingly, urea clearance is calculated in two different ways, depending upon the rate of flow of urine, i.e. as standard urea clearance and maximum urea clearance.

Standard Urea Clearance

Standard urea clearance is calculated with standard rate of urine flow (approximately 1 mL/min), i.e. when the value of urine volume is below 2 mL/min:

$$\text{Standard urea clearance (mL/min)} = \frac{U}{B} \times \sqrt{V},$$

where U and B are concentrations of urea (mg/100 mL) in urine and blood, respectively, while $\sqrt{V}$ is square root of the volume of urine excreted (mL/min). Mean value for standard urea clearance is 54 mL/min.

Maximum Urea Clearance

Maximum urea clearance is calculated when volume of urine excreted is 2 mL/min or more. It is calculated by putting the actual value of V in the above equation, instead of $\sqrt{V}$.

$$\text{Maximum urea clearance (mL/min)} = \frac{U}{B} \times V$$

Mean value for maximum urea clearance is 75 mL/min.

As discussed above, urea clearance depends upon the rate of urine flow and that blood urea concentration is affected by dietary protein, urea clearance test is not preferred to determine GFR.

Creatinine Clearance

Creatinine is produced endogenously from creatine and gains access to the urine by glomerular filtration. Its clearance is not affected by the volume of urine flow. Also, serum creatinine concentration is not affected by diet. Thus, creatinine clearance is preferred as a measure of GFR. Normal value for creatinine clearance is 95–105 mL/min.

Creatinine clearance (per 1.73 m^2 surface area) is low at birth (35–40 mL/min) and reaches the normal adult value (80–120 mL/min) by 6 months of age. It further declines with age. Creatinine clearance is increased by 20% during pregnancy.

PRACTICAL EXERCISES

1. Estimate blood and urine creatinine and calculate creatinine clearance.
2. Estimate blood and urine urea and calculate urea clearance.

Important Questions

1. Describe various functions of the kidney.
2. Discuss kidney function tests.
3. What are normal constituents of urine? Describe conditions in which their levels are increased.
4. What are abnormal constituents of urine? Discuss various conditions in which they are excreted.
5. Define clearance. Describe different types of clearance to assess kidney function.
6. Write notes on:
    - i. Polyuria
    - ii. Abnormal constituents of urine
    - iii. Renal glycosuria
    - iv. Ketonemia
    - v. Ketonuria
    - vi. Physiological proteinuria
    - vii. Renal clearance
    - viii. Creatinine clearance
    - ix. Maximum urea clearance
    - x. Standard urea clearance

Chapter

47

Thyroid Function Tests

The thyroid is a gland found in the neck. Its main function is to make thyroid hormones, the chemicals which are released into the bloodstream.

THYROID HORMONES

Thyroid makes three hormones that it secretes into the bloodstream. Two of these hormones called **thyroxine** (T_4) and **triiodothyronine** (T_3), increase body's metabolic rate. The third hormone, calcitonin, helps to control the amount of calcium in the blood.

In order to make $\mathbf{T_3}$ and $\mathbf{T_4}$, the thyroid gland needs iodine, a substance found in the food. It is taken up from bloodstream into the thyroid gland. In the thyroid gland, it then undergoes a number of different chemical reactions which result in the production of T_3 and T_4. T_4 is called so because it contains four atoms of iodine. T_3 contains three atoms of iodine. In the cells and tissues of the body most T_4 is converted to T_3. T_3 is the more active hormone; it influences the activity of all the cells and tissues of the body.

Regulation of Thyroid Activity

The activity of the thyroid is controlled by hormones produced by two parts of the brain, the hypothalamus and the pituitary.

- The **hypothalamus** receives input from the body about the state of many different bodily functions. When the hypothalamus senses levels of T_3 and T_4 are low, or that the body's metabolic rate is low, it releases a hormone called thyrotropin-releasing hormone (TRH).
- TRH travels to the **pituitary** via the connecting blood vessels and stimulates the pituitary to secrete thyroid-stimulating hormone (TSH). TSH is released from the pituitary into the bloodstream and travels to the thyroid gland. Here, TSH stimulates cells, within the thyroid, to make more T_3 and T_4. T_3 and T_4 are then released into the bloodstream where they increase metabolic activity in the cells.

THYROID FUNCTION TESTS

Thyroid function tests (TFTs) are a collective term for **blood tests** used to check the function of the thyroid. These tests are requested if a patient is thought to suffer from **hyperthyroidism** (overactive thyroid) or **hypothyroidism** (underactive thyroid), or to monitor the effectiveness of either thyroid-suppression or hormone replacement therapy. It is also requested routinely in conditions linked to thyroid disease, such as atrial fibrillation and anxiety disorder. Thyroid function tests can also be done to:

- Monitor treatment with thyroid replacement medicine for people who have hypothyroidism.
- Check thyroid gland function in people who are being treated for hyperthyroidism.
- Screen newborn babies for inherited problems with the thyroid.

HYPERTHYROIDISM

Hyperthyroidism, often called **overactive thyroid** and sometimes **hyperthyreosis**, is a condition in which the thyroid gland produces and secretes excessive amounts of the free (not protein bound circulating in the blood) thyroid hormones, T_3 and/or T_4.

A variety of conditions can cause hyperthyroidism. Graves' disease (an autoimmune disorder) is the most common. Other causes of hyperthyroidism include excess iodine (iodine is needed to make T_4 and T_3), inflammation of the thyroid gland (thyroiditis causes T_4 and T_3 to leak out of the gland), tumors of the ovaries or testes, benign tumors of the thyroid or pituitary gland or taking large amount of thyroxine (through dietary supplements or medication).

Graves' Disease

Graves' disease is the most common form or cause of hyperthyroidism. In this condition antibodies stimulate the thyroid to secrete too much hormone, the eyes may look enlarged because the eye muscles swell and push the eye forward. This symptom is called exophthalmos.

Some patients have swelling of the front of the neck from an enlarged thyroid gland. It occurs more often in women and tends to run in families. Other symptoms include weakness, irregular heartbeat, difficulty in sleeping, itching, hair loss, nausea and vomiting, and breast development in men.

Thyrotoxicosis

Hyperthyroidism is one cause of thyrotoxicosis, the hypermetabolic clinical syndrome, which occurs when there are elevated serum levels of T_3 and/or T_4. Thyrotoxicosis can also occur without hyperthyroidism. Some people develop thyrotoxicosis due to inflammation of the thyroid gland (thyroiditis) which can lead to excessive release of thyroid hormone already stored in the gland (without the accelerated hormone production that characterizes hyperthyroidism). Thyrotoxicosis can also occur after ingestion of excessive amounts of exogenous thyroid hormone in the form of thyroid hormone supplements.

Thyrotoxicosis or hyperthyroidism may be asymptomatic but when it is not, symptoms are due to an excess of thyroid hormone. If there is too much thyroid hormone, every function of the body tends to speed up. Some of the **symptoms of hyperthyroidism** may be nervousness, irritability, increased perspiration, heart racing, hand tremors, anxiety, difficulty sleeping, thinning of the skin, fine brittle hair and muscular weakness, especially in the upper arms and thighs, unintended weight loss and low serum cholesterol. For women menstrual flow may lighten and menstrual periods may occur less often. **Major clinical signs** include weight loss (often accompanied by an increased appetite), anxiety and intolerance to heat, hair loss (especially of the outer side of the eyebrows), muscle aches, weakness, fatigue, hyperactivity, irritability, hyperglycemia, polyuria, polydipsia, delirium, tremor and sweating.

The first step to **diagnose hyperthyroidism** is a complete history and physical examination, which can reveal common symptoms such as weight loss, rapid pulse, elevated blood pressure, protruding eyes and/or an enlarged thyroid gland (which can appear either symmetrical or one-sided). Other tests may be performed to further evaluate the diagnosis. These include **serum cholesterol** (cholesterol levels vary with the metabolic rate, the rate at which cells use energy. In hyperthyroidism, cholesterol can be low due to the elevated metabolic rate), **T_4 and T_3, TSH** (a hormone produced by the hypothalamus that stimulates the thyroid gland to produce thyroid hormones. When thyroid hormones levels are normal or high, TSH should not be elevated).

HYPOTHYROIDISM

Hypothyroidism, often called **underactive thyroid** or **low thyroid** and sometimes **hypothyreosis**, is a common endocrine disorder in which the thyroid gland does not produce enough thyroid hormone.

- **Iodine deficiency** is the most common cause of primary hypothyroidism and endemic goiter worldwide.
- In areas of the world with sufficient dietary iodine, hypothyroidism is most commonly caused by the autoimmune disease, **Hashimoto's thyroiditis** (chronic autoimmune thyroiditis). Hashimoto's disease may be associated with a goiter. It is characterized by infiltration of the thyroid gland with T lymphocytes and autoantibodies against specific thyroid antigens such as thyroid peroxidase, thyroglobulin and the TSH receptor. Autoimmune thyroiditis is associated with other immune-mediated diseases such as diabetes mellitus type I, pernicious anemia, myasthenia gravis, rheumatoid arthritis, etc.
- Hyperthyroidism due to certain types of **thyroiditis** can eventually lead to hypothyroidism (lack of thyroid hormone), as the thyroid gland is damaged.
- Also, **radioiodine treatment of Graves' disease** often eventually leads to hypothyroidism. Such hypothyroidism may be treated by regular thyroid hormone testing and oral thyroid hormone supplementation.

Hypothyroidism can cause a number of symptoms such as tiredness, poor ability to tolerate cold and weight gain. In children, hypothyroidism leads to delays in growth and intellectual development, called **cretinism** in severe cases. Cretin children are dwarf and mentally retarded.

The diagnosis of hypothyroidism, when suspected, can be confirmed with blood tests measuring TSH and thyroxine levels.

BIOCHEMICAL EVALUATION OF THYROID FUNCTIONS

Biochemical tests to establish if there is any thyroid dysfunction, include measurement of thyroid hormones (T_3 and T_4) and TSH in serum. Other tests include radioactive iodine uptake, protein bound iodine, serum cholesterol, etc.

Thyroid Hormones

The thyroid function test panel typically includes thyroid hormones such as thyroid-stimulating hormone (TSH, thyrotropin), and thyroxine (T_4) and triiodothyronine (T_3). Measurement of the concentration of T_4 and T_3 in the serum is used in confirming the diagnosis of hyperthyroidism or hypothyroidism.

Thyroxine

Total thyroxine is rarely measured, having been largely superseded by free thyroxine test. It is generally elevated in hyperthyroidism and decreased in hypothyroidism. It is usually slightly elevated in pregnancy, secondary to increased levels of thyroid binding globulin (TBG). Serum T_4 value in a normal person ranges from 4.5 to 12.5 mg/dL. In hyperthyroidism, serum T_4 concentration is increased to >12 mg/dL, whereas in hypothyroidism the value is <5 mg/dL. Estimation of free thyroxine (FT_4), however, provides more reliable information than does total T_4. Free T_4 is generally elevated in hyperthyroidism and decreased in hypothyroidism.

Triiodothyronine

Total triiodothyronine (total T_3) is rarely measured, having been largely superseded by free T_3 test. Total T_3 is generally elevated in hyperthyroidism and decreased in hypothyroidism. Normal value for serum T_3 varies between 86 and 187 ng/dL. Serum T_3 concentration is low at birth. The value rises to near adult level in early childhood and then is maintained up to 30 years of age, after which it again falls.

Measurement of reverse T_3 (rT_3, a catabolic product of T_4) may also be helpful in some cases, e.g. in low T_3 syndrome where rT_3 concentration is increased. Free T_3 is generally elevated in hyperthyroidism and decreased in hypothyroidism.

Thyroid-stimulating Hormone

Thyroid-stimulating hormone (TSH) is the most sensitive test for thyroid hormone function. TSH is produced in the pituitary gland. The production of TSH is controlled by thyrotropin releasing hormone (TRH) which is produced in the hypothalamus. TSH levels may be suppressed by excess free T_3 or free T_4 in the blood. Reference value for serum TSH concentration is in the range of 0.3–5.0 mIU/L. Measurement of TSH is most useful and sensitive for primary hypothyroidism.

Thyroxine-binding Globulin

An increased thyroxine-binding globulin results in an increased total thyroxine and total triiodothyronine without an actual increase in hormonal activity of thyroid hormones. Reference range is 12–30 mg/L.

Thyroid Hormone Uptake

Thyroid hormone uptake (T_{uptake} or $T_{3\ \text{uptake}}$) is a measure of the unbound thyroxine-binding globulin in the blood, i.e. the TBG that is unsaturated with thyroid hormone. Unsaturated TBG increases with decreased levels of thyroid hormones. It is not directly related to triiodothyronine, despite the name $T_{3\ \text{uptake}}$.

Stimulation/Suppression Tests

TRH Stimulation Test

Thyrotropin releasing hormone (TRH) stimulation test is used to assess the functional state of the TSH secretory mechanism. Serum TSH is assayed before and 20, 30 and 60 minutes after a standard dose of TRH. In normal individuals, serum TSH peaks are observed in 30 minutes and values generally range between 5 and 30 mIU/L. A normal response excludes the possibility of thyrotoxicosis. In hyperthyroidism, no significant rise in TSH occurs because elevated thyroid hormones inhibit TSH production. In primary hypothyroidism, TSH response to TRH is exaggerated and is of diagnostic significance. It is often useful in differentiating between pituitary and hypothalamic lesion as the cause of secondary hypothyroidism.

Thyroid Suppression Test

Normally, exogenous thyroid hormone suppresses pituitary TSH secretion, resulting in a decrease in RAIU. An abnormal suppression test is observed in hyperthyroidism, irrespective of the underlying cause. A normal suppression test excludes the diagnosis of hyperthyroidism.

Autoimmune Profile

Antibodies against thyroid peroxidase (**anti-TPO antibodies**) may be detected in the serum of some hypothyroid patients. In Graves' disease, long-acting thyroid stimulator (**LATS**) is detectable in serum.

Other Tests

Radioactive Iodine Uptake

As iodine plays an important role in the metabolism of the thyroid gland, radioactive iodine uptake (**RAIU**) is used for thyroid function studies. Usually, 10 µci (microcurie) of sodium or potassium iodide is administered orally (^{125}I delivers low radiation dose, hence it is preferred over ^{131}I). The radioactivity mixes uniformly with the endogenous iodide in the extracellular fluid. Percentage of the iodide entering and leaving the extracellular space per unit time, which is accumulated by the thyroid in the steady state, is assessed. RAIU is usually measured 24 hours after the administration of the isotope. Normally, 24 hours RAIU uptake is 5–30% of the administered dose. Values above the normal range indicate hyperfunctioning of the gland and are useful in the diagnosis of hyperthyroidism. RAIU is also used as a part of thyroid suppression test.

Protein-bound Iodine

Serum protein-bound iodine (PBI) is an indirect measure for assessing thyroid function since about 90% of the iodine in serum is bound to thyroxine. PBI value in a normal subject ranges from 4 to 8 mg/100 mL. In hypothyroidism, PBI value varies from 0.2 to 2.5 mg/100 mL, whereas in hyperthyroidism PBI value ranges from 9 to 18 mg/100 mL.

Basal Metabolic Rate (BMR)

It is a measurement of oxygen consumption in the basal state. BMR is raised in hyperthyroidism and is reduced in hypothyroidism.

Serum Cholesterol

Increase in serum cholesterol is observed in hypothyroidism, whereas it is decreased in hyperthyroidism or thyrotoxicosis.

Serum Calcium

Hyperthyroidism may result in increased plasma calcium along with increased plasma alkaline phosphatase activity.

Glucose Tolerance Test (GTT)

It is sometimes abnormal in patients with hyperthyroidism who may show a diabetic type of response.

Important Questions

1. Describe thyroid function tests.
    - i. Hyperthyroidism
    - ii. Hypothyroidism
    - iii. Graves' disease
    - iv. Biochemical evaluation of thyroid functions
2. Write notes on:
    - i. Diastase
    - ii. 17-Ketosteroids
    - iii. VMA

Chapter

48

Quantitative Estimation of Some Biochemical Constituents in Urine

ESTIMATION OF 24 HOURS URINARY PROTEINS

The glomeruli act as an ultrafilter for plasma proteins. The degree to which an individual protein is filtered depends upon its molecular size, ionic charge and plasma concentration. In addition, the proportion of individual protein excreted in urine depends upon the extent of its reabsorption by the proximal renal tubules, which is also inversely proportional to molecular size. Normally, <150 mg of proteins are excreted in urine per day. Excretion of >300 mg/day constitutes macroproteinuria. Due to such a low concentration, determination of protein in urine is much more difficult than in serum. Large sample to sample variations, variable composition of proteins, high concentration of nonprotein interfering substances and high inorganic ion content add to the problem of the estimation of urinary proteins.

Most commonly used method for the estimation of proteins in a biological sample is called biuret method (*see* Chapter 23).

As the concentration of proteins in urine is very low, the biuret method, used for serum, is modified for the estimation of proteins in urine and is referred to as modified biuret method.

Modified Biuret Method

Principle

In this method, proteins present in urine are concentrated by precipitation with trichloroacetic acid (TCA) or ethanolic-HCl-phosphotungstic acid (Tsuchiya's reagent) and re-dissolved in an alkaline solution (I N NaOH). With the biuret reagent, colored complex is formed, which is measured at 540 nm. The intensity of the violet color produced is proportional to the concentration of proteins.

Reagents

1. 10% TCA
2. Distilled water (DW)
3. Biuret reagent (quantitative)
4. Working standard: 7 g% (prepared with pooled serum)

Procedure

1. Measure the volume of 24 hours timed urine collection, which must be fresh.
2. Aliquot 30 mL in a large centrifuge tube.
3. Centrifuge at 1500 × g for 15 min. The supernatant may be stored at 4°C for later analysis (if required).

Take 3 test tubes, and label them as blank (B), standard (S) and test (T). Add urine, distilled water, standard protein solution and biuret reagent as shown in Table 48.1.

Table 48.1 Preparation of test tubes for the estimation of proteins in urine by modified biuret test

	Test (T)	Standard (S)	Blank (B)
	2.5 mL urine	2.5 mL working standard	2.5 mL DW
10% TCA	2.5 mL	2.5 mL	2.5 mL
Centrifuge and discard the supernatant. Now, again proceed as follows:			
DW	2.5 mL	2.5 mL	2.5 mL
Biuret reagent	2.5 mL	2.5 mL	2.5 mL

Mix well and incubate at 37°C for 10 min and take the OD at 540 nm

Calculations

$$\text{Urinary proteins mg\%} = \frac{OD_T \times 7}{OD_S}$$

Normal Value

Normally, <150 mg of proteins are excreted in urine, in 24 hours.

Other Methods

1. Turbidimetric method
2. Dye binding method
3. Dipstick strips: It is a semiquantitative assessment.

Interpretation

Proteinuria

Proteinuria may be physiological (where only a small amount of protein may be excreted in urine for some time) or pathological (where large amount of protein may be excreted for prolonged duration).

- **Physiological:** Erect posture for long time, strenuous exercise, or during pregnancy.
- **Pathological: Causes may be grouped as**
    a. **Prerenal** causes, which include infection, fever, a heart disease, severe anemia, severe dehydration and increased intra-abdominal pressure, such as in ascites and tumors.
    b. **Renal** causes include diseases, such as nephrotic syndrome, glomerulonephritis, or urinary tract infection (UTI).
    c. **Postrenal** causes include obstruction in the urinary tract, may be due to stone, stricture or tumor.

ESTIMATION OF 24 HOURS URINARY UREA

Urea is the end product of protein catabolism. Urinary urea concentration can be reported in terms of urea or urea nitrogen. As the molecular weight of urea is 60 and that of urea nitrogen is 28, so concentration of urea = 2.14 × urea nitrogen. Reported value is in terms of urea, if urea standard is used while performing the test or in terms of urea nitrogen, if the urea nitrogen standard is used while performing the test.

Method of Estimation

As discussed in Chapter 23, urea is generally measured by diacetyl monoxime (DAM) method.

Principle

Urea reacts with diacetyl monoxime in hot acidic medium, in the presence of thiosemicarbazide and cadmium ions to form a pink-colored compound, which can be measured on a green filter (540 nm).

Reagents

1. **Thiosemicarbazide reagent**
2. **Diacetyl monoxime solution** (2%)
3. **Urea standard** (20 mg of urea nitrogen/100 mL)

Procedure

Dilute urine 1:10 in DW. Take three test tubes, label them as test (T), standard (S) and blank (B), and add various reagents as shown in Table 48.2.

Table 48.2 Preparation of test tubes for the estimation of urea in urine

	Test	Standard	Blank
Working reagent, mL	5.0	5.0	5.0
Dilute urine	0.05	–	–
20 mg/dL urea nitrogen standard, mL	–	0.05	–
DW, mL	–	–	0.05

Mix the contents properly and keep in a boiling water bath for 20 min. Cool the tubes and read the absorbance at 540 nm against blank.

Calculation

$$\text{Urine urea nitrogen, mg\%} = \frac{OD_T \times 20 \times 10}{OD_S}$$

Normal Value

24 hours normal urinary excretion of urea nitrogen = 12–15 g.

Other Methods

1. **Determination of urea nitrogen by Berthelot reaction method:** Urease splits urea into ammonia and carbon dioxide. The ammonia reacts with phenol, in the presence of hypochlorite, to form indophenol, which, with alkali gives a blue-colored compound that can be measured at 546 nm. The color of the reaction is stable for 12 hours.
2. **Determination by UV kinetic method:** Urease splits urea into ammonia and carbon dioxide. In the presence of ammonia, α-ketoglutarate and glutamate dehydrogenase, NADH is oxidized to NAD^+. The rate of decrease in OD is measured at the interval of 30 sec, at 340 nm, which is directly proportional to the urea concentration in the sample.

Interpretation

Urine urea estimations are carried out as a part of renal efficiency tests. A high concentration of urea shows that the kidney possesses a good concentrating power and is not found in presence of renal failure. In cases of increased blood urea due to pre-renal causes, urine urea may be quite high.

ESTIMATION OF 24 HOURS URINARY CREATININE

Creatinine is the anhydride form of creatine. It is a waste product that is excreted in urine. Along with serum creatinine, urinary creatinine is used in the calculation of creatinine clearance to get the idea of glomerular filtration rate (GFR). As mentioned in Chapter 23, creatinine is estimated by Jaffe reaction.

Principle

Creatinine reacts with alkaline picrate and forms orange-red complex. This is known as **Jaffe reaction**.

Reagents

1. 10% sodium tungstate
2. 2/3 N H_2SO_4
3. Saturated picric acid solution
4. Alkaline picrate reagent: Five mL of 10% NaOH is added to 25 mL of the saturated picric acid solution, just before use.
5. Working standard solution containing 20 mg/mL of creatinine.

Procedure

Dilute urine 1:100 with distilled water so that interfering substances get diluted and the values come within the range of Lambert-Beer law.

Take three test tubes, label them as blank (B), standard (S) and test (T), and add various reagents as shown in Table 48.3.

Table 48.3 Preparation of test tubes for the estimation of creatinine in urine

	B	S	T
Distilled water	3 mL	–	–
Standard (0.01 mg/mL)	–	3 mL	–
Dilute urine	–	–	3 mL
NaOH	1 mL	1 mL	1 mL
Picric acid	1 mL	1 mL	1 mL

Mix the contents and read optical density at 540 nm, after 15 min.

Calculation

$$\text{Urine creatinine (mg\%)} = \frac{A_T}{A_S} \times \begin{matrix}\text{Concentration}\\ \text{of standard}\end{matrix}$$

$$\begin{matrix}\text{Amount of creatinine in}\\ \text{3 mL of diluted urine}\end{matrix} = \frac{A_T \times 0.03}{A_S}$$

$$\begin{matrix}\text{Amount of creatinine in}\\ \text{100 mL of diluted urine}\end{matrix} = \frac{A_T \times 0.03 \times 100}{A_S \times 3}$$

$$\begin{matrix}\text{Amount of creatinine in}\\ \text{100 mL of normal urine}\end{matrix} = \frac{A_T \times 0.03 \times 100 \times 100}{A_S \times 3}$$

$$= \frac{A_T \times 100}{A_S}$$

Normal Urinary Excretion

1–2 g/day

Interpretation

It is of interest in pathological conditions related to muscular disorders. Increased levels may be seen in:

- Myasthenia gravis
- Muscular atrophies
- Different types of myositis
- Starvation
- Hyperthyroidism
- Fever associated with considerable muscle wasting.

24 HOURS URINARY URIC ACID

Uric acid is the end product of catabolism of purines. Renal handling of uric acid is complex and involves four sequential steps:

1. Glomerular filtration
2. Reabsorption in the proximal convoluted tubule
3. Secretion into the lumen, in the distal portion of the proximal tubule.
4. Further reabsorption in the distal tubule.

Net urinary excretion of uric acid is about 6 to 12% of the amount filtered.

Method of Estimation

Uric acid is estimated by using phosphotungstic acid, called phosphotungstic acid method or Caraway method. It is based on the reducing property of uric acid.

Principle

Uric acid reacts with phosphotungstic acid in the presence of sodium carbonate and reduces it to blue colored complex, absorbance of which is measured at 660 nm.

Procedure

1. **Dilute urine** 1:200. For this purpose, pipette 19.9 mL of distilled water in a test tube and add 0.1 mL of urine. Mix well.
2. **Dilute stock standard solution** (100 mg/dL) uric acid standard 1:200. Pipette 19.9 mL of distilled water in a test tube and add 0.1 mL of stock standard. Mix well.

Take 3 test tubes and label them as T (test), S (standard) and blank (B), and add various reagents as shown in Table 48.4.

Table 48.4 Preparation of test tubes for the estimation of uric acid in urine

	Test	Standard	Blank
Dilute urine, mL	3.0	–	–
Dilute standard, mL	–	3.0	–
Distilled water, mL	–	–	3.0
Sodium carbonate (10 g/dl), mL	1.0	1.0	1.0
Dilute phosphotungstic acid reagent, mL	1.0	1.0	1.0

Mix thoroughly and keep in dark for exactly 10 minutes. Read intensities at 660 nm (red filter).

Calculation

(a) Urine uric acid, mg/dL= $\dfrac{\text{OD}_\text{T} \times 100}{\text{OD}_\text{S}}$

(b) 24 hours uric acid excretion $= \dfrac{\begin{array}{c}\text{Urine uric acid}\\ \text{(mg/dL)}\end{array} \times \begin{array}{c}\text{24 hr urine}\\ \text{volume (ml)}\end{array}}{100}$

Reference Range

The quantity excreted in urine depends upon the purine content of the diet and normally the excretion rate is 0.5–1.0 g/24 hours.

Interpretation

Urine uric acid follows a characteristic course in gout, being low before the attack and increases during the attack. A consistently high excretion is found in leukaemia and in conditions in which there is increased metabolism of nucleoprotein without impairment of renal functions. Administration of cortisone or ACTH increases uric acid excretion.

ESTIMATION OF URINARY DIASTASE

A diastase is any one of a group of enzymes (α-, β-, or γ-amylase), which catalyzes the breakdown of starch into a mixture of the disaccharide maltose, the trisaccharide maltotriose and dextrins. Urine diastase is useful in diagnosing uncertain abdominal cases, especially when pancreatitis is suspected.

Principle

It is estimated by the Wohlgemuth method. In this method diminishing volumes of urine are used and the least volume which completely digests a certain amount of starch is used to find the diastatic index.

Reagents

1. **Stock starch solution:** 2 g of starch and 10 g of sodium chloride per 100 mL of solution.
2. **Working starch solution:** 0.1% solution.
3. **Iodine solution:** Approximately 0.02 N iodine solution.

Procedure

Adjust the pH of the urine so as to be just acid to litmus, using either strong sodium hydroxide solution or concentrate hydrochloric acid.

Put a series of test tubes as shown in Table 48.5.

Table 48.5 Preparation of test tubes for the diastase in urine

	1	2	3	4	5			
Diastatic index	2	4	6.7	10	14.3			
Urine (mL)	1.0	0.5	0.3	0.2	0.14			
Water (mL)	0.0	0.5	0.7	0.8	0.86			
	6	**7**	**8**	**9**	**10**	**11**	**12**	**13**
Diastatic index	20	28.6	40	50	67	100	133	167
Urine (1:10)	1.0	0.7	0.5	0.4	0.3	0.2	0.15	0.12
Water (mL)	0.0	0.3	0.5	0.6	0.7	0.8	0.85	0.88

Add 2 mL of 0.1% starch solution to each tube and place the tubes in water bath at 37°C for 30 min. After removing the tubes from the water bath, immediately place in cold water for a minute.

Now, set the tubes in a rack and add the iodine solution, slowly, drop by drop, to each tube and observe carefully whether any blue color is produced. The smallest volume of urine which does not give any blue color is noted. This tube may show a reddish color due to presence of erythrodextrin.

Note: The dilution of urine is continued until a point is reached at which digestion is incomplete. With the high values of diastatic index it may be necessary to use 1:100 or 1:1000 dilution, occasionally.

Calculation

Diastatic index is defined as the number of mL of 0.1% starch digested by 1 mL of urine at 37°C in 30 min.

Diastatic index = 2, if smallest volume of urine is not giving a blue color.

So, say if tube number 11 is the first one to show a blue color with iodine, the least amount of urine just able to digest 2 mL of 0.1% starch is in tube 10, that is 0.3 mL of 1:10 urine or 0.03 mL of urine.

Thus, diastatic index = = 67 units

The estimation is carried out on a 24 hours urine sample, which may be preserved with a little quantity of toluene, as preservative. In urgency, such as in acute abdominal conditions, the test may be done at once, on a random urine specimen.

Reference Interval

Urine diastase (or amylase) normally corresponds to a diastatic index from 5 to 20 units. This applies to a 24-hour specimen.

In a random sample, a wide reference interval is found, values reaching up to 50 units with concentrate urine.

Interpretation

Variation in urinary amylase reflect alterations in serum amylase as long as kidneys are functioning normally. In a renal disease, urine amylase may be low. It may be increased in acute pancreatitis, intestinal obstruction, perforated peptic ulcer.

ESTIMATION OF 17-KETOSTEROIDS IN URINE

A **ketosteroid**, or an **oxosteroid**, is a steroid in which a hydrogen atom has been replaced with a ketone (C=O) group. A **17-ketosteroid** is a ketosteroid in which the ketone is located specifically at the C17 position. Examples of 17-ketosteroids include androstenedione, androsterone, dehydroepiandrosterone, epiandrosterone, etc.

17-Ketosteroids are endogenous steroid substances that form when the body breaks down male steroid sex hormones, called androgens and other hormones, released by the adrenal glands in males and females, and by the testes in males. In men, about one-third of the total 17-ketosteroids in urine represent metabolites of testosterone secreted by testes and the remaining two-thirds from the steroids produced by adrenal glands. In women, total 17-ketosteroids in the urine are mainly derived from the adrenals.

Method of Estimation

Method of preparation of a suitable urine extract and the determination of total urinary 17-ketosteroids (17-KS) is commonly done by the modification of the Zimmermann reaction. Accordingly, acid cleavage of glucuronic and sulfuric acid conjugates of 17-KS is followed by extraction, washing with alkali and finally, development of the reddish-purple color, which is read at 520 nm.

Besides, gas chromatography with mass spectrometry though is very specific, HPLC is very commonly used.

Note: Collection of urine specimen:

1. **24 hours urine specimen:** Add 25 mL of 50% acetic acid to collection vessel as a preservative. Note patient's age and total urine volume collected over a period of 24 hours.
2. **Random urine** (in children only): Random urine sample is collected and the urine pH is adjusted to 1–5 with 50% acetic or hydrochloric acid.

For better interpretation, it is advised to estimate urine creatinine also.

Normal Value

In men, 10–25 mg/day; Women 6–14 mg/day.

Interpretation

- **Decreased concentration** of 17-KS is seen in men with primary or secondary hypogonadism, and in women with pituitary hypoadrenalism.
- **Increased level is found** in testicular tumors, adrenal hyperplasia and adrenal carcinoma.
- Many drugs interfere with 17-KS assay. For example, chlorpromazine, penicillin, nalidixic acid, spironolactone, etc. produce positive interference. On the other hand, chlordiazepoxide, progestational agents and reserpine, etc. produce negative interference.

ESTIMATION OF VANILLYLMANDELIC ACID IN URINE

Vanillylmandelic acid (**VMA**) is an end-stage metabolite of the catecholamines, epinephrine and norepinephrine. It is formed by the action of catechol-o-methyl transferase and MAO (monoamine oxidase) via intermediary metabolites.

VMA is found in the urine along with other metabolites, including homovanillic acid (HVA), metanephrine and normetanephrine. Its excretion represents an average of 40 to 50% of the urinary excretion production of norepinephrine and epinephrine. In timed urine tests the quantity excreted (usually per 24 hours) is assessed along with creatinine clearance.

It is preferred that the patient should be off medications for three days prior to collection. However, common antihypertensives (diuretics, ACE inhibitors, calcium channel blockers, alpha and beta blockers) cause minimal or no interference. Patient should avoid alcohol, coffee, tea, tobacco (including use of nicotine patch), bananas, citrus fruits and strenuous exercise prior to collection.

HPLC is very commonly used method for the estimation of VMA though gas chromatography with mass spectrometry is highly specific method for its detection.

Normal Value

It is advised to represent the value of urine VMA against creatinine concentration. It is decreased with age.

Age	mg/g creatinine
0–1 year	<27
1–2 yrs	<18
2–4 yrs	<13
5–9 yrs	<8.5
10–14 yrs	<7
>15 yrs	2–7

Interpretation

Urinary VMA is elevated in patients with tumors that secrete catecholamines. Its analysis is commonly used to diagnose an adrenal gland tumor called pheochromocytoma (a tumor of catecholamine-secreting chromaffin cells). It may also be used to diagnose neuroblastoma and to monitor treatment of these conditions.

Coffee, tea, banana, cocoa, chocolate, vanilla, etc. increase urinary excretion of catecholamines.

PRACTICAL EXERCISES

1. Estimation of proteins in a 24-hour urine sample.
2. Estimation of urea in a 24-hour urine sample.
3. Estimation of creatinine in a 24-hour urine sample.
4. Estimation of uric acid in a 24-hour urine sample.
5. Estimation of diastase in urine.
6. Estimation of 17-ketosteroids in urine.
7. Estimation of VMA in urine.

Important Questions

1. Discuss:
    i. Significance of the estimation of protein in urine
    ii. Significance of the estimation of urea in urine
    iii. Significance of the estimation of uric acid in urine
    iv. Significance of the estimation of creatinine in urine
2. Write notes on:
    i. Diastase
    ii. 17-Ketosteroids
    iii. VMA

Chapter

49

Minerals

Minerals are inorganic substances required by the body in small amounts for a variety of functions. These include the formation of bone and teeth; as essential constituents of body fluids and tissues; as components of enzyme systems and for normal nerve function. Some minerals are needed in larger amounts than others, e.g. calcium, phosphorus, magnesium, sodium, potassium and chloride. Others are required in smaller quantities and are sometimes called trace minerals, e.g. iron, zinc, iodine, fluoride, selenium and copper.

Understanding the range and variability of the content of these minerals in biological samples can provide insight into the relationships between mineral content and the health of individuals. In particular, abnormal mineral content may serve as an indicator of illness.

GENERAL METHODS OF ESTIMATION

Depending upon their concentration in the biological fluid and the sensitivity of the method, electrolytes and minerals can be estimated by various methods, which include:

1. Colorimetry (*see* Chapter 22)
2. Flame photometry (*see* Chapter 24)
3. Ion-selective electrodes (*see* Chapter 24)
4. Atomic absorption spectrophotometry (*see* Chapter 41)

CALCIUM

The average adult store of calcium is approximately 1–2 kg. The vast majority (99%) resides in the skeleton. Only a fraction of the stored calcium is present in extracellular fluid and available for use in the form of ionized calcium. Ionized calcium is tightly regulated by PTH.

Calcium enters the extracellular fluid through absorption from the gut and resorption from bone. It is removed through secretion into the gastrointestinal tract and urine as well as losses in sweat and deposition in bone.

Calcium ions play a vital role in the physiology and biochemistry of organisms and the cell as electrolytes. They play an important role in signal transduction pathways, where they act as a second messenger, in neurotransmitter release from neurons, in contraction of muscle and in fertilization. Many enzymes require calcium ions as a cofactor. Calcium ions outside cells are also important for maintaining the potential difference across excitable cell membrane as well as proper bone formation.

Urinary calcium level reflects dietary intake. In an average adult urine sample collected over 24 hours, 100–250 mg of calcium is excreted. Calcium excretion is heavily influenced by sodium excretion. Low-sodium diets tend to decrease calcium excretion and vice versa.

Normal Value

- **Serum calcium:** 8.5–10.5 mg/100 mL.
- **Urinary calcium:** 100–700 mg/24 hour.

Alterations in Serum Calcium

Hypocalcemia

Hypocalcemia may result, primarily, from medical problems or treatments, including renal failure, surgical removal of the stomach and use of certain medications (such as diuretics). Inadequate calcium or vitamin D intake may also result in hypocalcemia, often caused by inadequate secretion of parathyroid hormone or defective receptors, to it, in cells.

Symptoms of hypocalcemia include numbness and tingling in the fingers, muscle cramps and convulsions, lethargy, poor appetite and abnormal heart rhythms (potentially causing tetany and defects in cardiac conduction).

Over the long term, inadequate calcium intake causes osteopenia, which if untreated, can lead to osteoporosis. The risk of bone fractures increases, especially in older individuals. Calcium deficiency can also cause rickets, though it is more commonly associated with vitamin D deficiency

Hypercalcemia

Excessively high levels of calcium in the blood is known as hypercalcemia. It can cause renal insufficiency, vascular and soft tissue calcification, hypercalciuria (high levels of calcium in the urine) and kidney stones.

Although very high calcium intakes have the potential to cause hypercalcemia, it is most commonly associated with excessive vitamin D intake or excessive secretion of parathyroid hormone. It can also occur due to the bone destruction that occurs when tumours metastasize to bone. This results in deposition of calcium salts into the heart, the blood vessels and the kidneys. Symptoms include anorexia, nausea, vomiting, memory loss, confusion, muscle weakness, increased urination, dehydration and metabolic bone disease. High calcium intake can also cause constipation.

Alterations in Urinary Calcium

An abnormally high amount of urinary calcium is called **hypercalciuria** and an abnormally low amount is called **hypocalciuria**.

Hypercalciuria

Elevated urine calcium (>300 mg/24 hr) is often a sign of an overactive parathyroid gland. Any disease, causing increase in serum calcium can lead to increase in urine calcium. In addition to hyperparathyroidism, other diseases include multiple myeloma (or any osteolytic neoplasm), osteoporosis, vitamin D overdose, renal tubular acidosis, hyperthyroidism, Paget's disease and sarcoidosis. Drugs containing calcium (such as some antacids) and calcium supplements can lead to direct increase in urine calcium. The diuretic spironolactone can also cause increase in urine calcium since it is given as a calcium salt and appears to decrease tubule reabsorption of calcium. Androgens, such as nandrolone, and treatment with growth hormone can also cause increase in urine calcium. Acetazolamide and systemic corticosteroids are also associated with increased calcium excretion.

Hypocalciuria

Some drugs can decrease urine calcium. Decreased urine calcium is also seen in hypoparathyroidism, pseudohypoparathyroidism (a lack of response to PTH rather than decreased secretion of PTH), rickets, hypothyroidism, steatorrhea and nephrosis. Another cause of low urine calcium is familial hypocalciuric hypercalcemia, also known as familial benign hypercalcemia.

ESTIMATION OF SERUM AND URINARY CALCIUM

a. **Ionized calcium:** Ion-selective electrodes

b. **Total calcium:**

   1. **Titration**
   2. **Colorimetric**
   3. **Atomic absorption spectrophotometry**
   4. **Flame photometry**

ESTIMATION OF SERUM CALCIUM

Titrimetric Method

Principle

Calcium is precipitated as oxalate and is titrated with standard potassium permanganate solution.

Reagents

1. **Ammonium oxalate solution:** 4%
2. **Dilute ammonia solution:** 2 mL of liquor ammonia with 98 mL of water
3. **H_2SO_4:** 1 N
4. **Potassium permanganate solution** (0.01 N)**:** The solution is standardized before use. For its standardization, 25 mL of 0.01 N oxalic acid is transferred to an Erlenmeyer flask. To it, add one mL of concentrated H_2SO_4 and warm the contents to about 70°C. Titrate against $KMnO_4$ solution, till the faint pink color remains. The normality of $KMnO_4$ solution = 25 × 0.01/titre value (mL).
5. **Oxalic acid** (0.01 N)**:** Sodium oxalate is dried in an oven at 100–105°C for 12 hours. Exactly, 0.67 g of it is dissolved in redistilled water. Thereafter, 5 mL of concentrated H_2SO_4 is added to it and the solution is made up to 1 L after it has cooled down.

Procedure

Two mL of sample is taken in a 15 mL centrifuge tube. Add 2 mL of distilled water and 1 mL of ammonium oxalate solution. Mix thoroughly and leave overnight. Again, the contents are mixed and centrifuged for 5 min at 1500 rpm. The supernatant is poured off and the centrifuge tube is drained by inverting the tube for 5 min on a rack (care should be taken not to disturb the precipitate). The mouth of the centrifuge tube is wiped with a piece of filter paper. The precipitate is stirred and the sides of the tube are washed with 3 mL of dilute ammonia. It is centrifuged again and drained, as before. The precipitate is washed once more with dilute ammonia to ensure the complete removal of ammonium oxalate. The precipitate is dissolved in 2 mL of 1 N $H_2SO_4^-$. The tube is heated by placing it in a boiling water bath for 1 min and titrated against 0.01 N $KMnO_4$ solution to a definite pink color that persist for at least 1 min.

Calculation

1 mL of 0.01 N $KMnO_4$ is equivalent to 0.2004 mg of calcium. Hence,

$$\text{mg of calcium/100 mL serum} = (x - b) \times 0.2004 \times (100/2),$$

where

x = Volume (in mL) of 0.01 N $KMnO_4$ required to titrate the sample, and

b = Volume (in mL) of 0.01 N $KMnO_4$ required to titrate 2 mL of H_2SO_4 (blank). If the normality of $KMnO_4$ is a, the value obtained in the above formula should be multiplied by the factor, $a/0.01$

Note: Potassium permanganate solution needs to be frequently standardized.

Colorimetric Method

Principle

Calcium forms a colored complex with **o-cresolphthalein complexone** (OCPC), which is made more specific in the presence of 8-quinolinol.

Reagents

1. Standard $CaCl_2$: Dissolve 25 mg of anhydrous $CaCO_3$ in dilute HCl, and make up to 100 mL with water. Dilute 1 mL to 10 mL, to get 10 µg calcium/mL.
2. Ammonia/ammonium chloride buffer (pH 10.5): Dissolve 0.24 g of NH_4Cl in 100 mL of 5% NH_4OH solution (V/V).
3. O-Cresolphthalein solution (0.1%): 100 mg of o-cresolphthalein is dissolved in 28 mL of ammonia/ammonium chloride buffer, and diluted to 100 mL with water.
4. 8-quinolinol: 1% solution in absolute ethanol.

Procedure

Different aliquots of the standard $CaCl_2$ (2–10 µg) are taken and the volume is made up to 5.5 mL with water. Add 1 mL of 8-quinolinol solution and 2.5 mL of ammonia/ammonium chloride buffer, and the volume is adjusted to 9.0 mL. The contents are finally treated with 1 mL of o-cresolphthalein solution. After addition of each reagent, as mentioned above, the contents are thoroughly mixed. Water blank in the place of $CaCl_2$ solution is simultaneously carried throughout the procedure. Standards are read against blank at 565 nm. Take readings after 5 min. The color is stable up to 3 hours.

Calculation

$$\text{Serum calcium (mg/dL)} = \frac{OD_T \times 10}{OD_S}$$

ESTIMATION OF URINARY CALCIUM

Calcium salts, such as calcium oxalate, precipitate in urine specimen after collection. So, 24 hours urine collection must be done with 20–30 mL of 6N HCl in the container.

Procedure

It is exactly the same as for serum calcium determination but instead of serum use 24 hours urine sample.

Calculation

$$\text{Urine calcium (mg/dL)} = \frac{OD_T \times 10}{OD_S}$$

$$\text{24 hours urinary excretion of calcium} = \frac{\text{Urine calcium (mg/dL)} \times \text{Urine volume}}{100}$$

For higher values, use dilute urine (1:2 or 1:5) and then calculate urinary calcium by using the appropriate dilution factor.

INORGANIC PHOSPHATES

In biological systems, phosphorus is found as a free phosphate ion in solution and is called **inorganic phosphate**, to distinguish it from phosphates bound in various phosphate esters. Inorganic phosphate is, generally, denoted as $\mathbf{P_i}$ and at physiological (homeostatic) pH, primarily, consists of a mixture of HPO_4^{2-} and $H_2PO_4^-$ ions.

Phosphate is useful in animal cells as a buffering agent. Phosphate salts that are commonly used for preparing buffer solutions at cell pH, include monosodium and disodium phosphates, and the corresponding potassium salts.

An important occurrence of phosphates in biological systems is as the structural material of bone and teeth. These structures are made of crystalline calcium phosphate in the form of hydroxyapatite.

Organic phosphates, in particular adenosine triphosphate (ATP), take part in a series of reversible reactions involving phosphoric acid, lactic acid, glycogen and other substances. It is also a component of nucleic acids, nucleotides, phospholipids and of many enzymes.

Phosphate is vital for energy metabolism, muscle contraction, nerve signalling, intracellular signalling and electrolyte transport. Phosphate is an important buffer, particularly in the urine, and contributes to the maintenance of acid–base status.

Serum phosphate is helpful in the diagnosis and management of a variety of disorders including bone, parathyroid and renal diseases.

Kidneys help controls the amount of phosphate in the body. Extra phosphate is filtered by the kidneys and passes out of the body in the urine. If there is not enough phosphate, less is found in the urine.

Normal Level

- **Serum**: 2.5–4.5 mg/dL
- **Urine:** 0.4–1.3 g/ 24 hours urine

Alteration in Serum Phosphate Level

Serum phosphate concentrations are dependent on meals and variation in the secretion of hormones, such as parathyroid hormone (PTH) and may vary widely. Many medications can affect phosphorus levels, including antacids, vitamin D supplements (when taken in excess), intravenous glucose and medications that contain sodium phosphate.

Hyperphosphatemia

Hyperphosphatemia means having too much phosphorus in blood. Hyperphosphatemia is usually secondary to an inability of the kidneys to excrete phosphate. Other factors may relate to increased intake or a shift of phosphate from the tissues into the extracellular fluid.

The most acute problem associated with rapid elevations of serum phosphate levels is hypocalcemia with tetany, seizures and hypotension. Soft tissue calcification is also an important long-term effect of high phosphorus levels.

High phosphate levels may indicate:

- Hypoparathyroidism (low levels of the parathyroid hormone)
- Cardiovascular disease (disorders of the blood vessels or heart)
- Liver disease
- Kidney failure
- Osteoporosis (condition that causes brittle and weak bones)

Hypophosphatemia

Hypophosphatemia is having too little phosphorus in blood. Hypophosphatemia may have 4 general causes, i.e. shift of phosphate from extracellular to intracellular, renal phosphate wasting, loss from the gastrointestinal tract and loss from intracellular stores.

Hypophosphatemia is relatively common in hospitalized patients. Serum concentration of phosphate between 1.5 and 2.4 mg/dL may be consider moderately decreased and are not usually associated with clinical signs and symptoms. Levels below 1.5 mg/dL may result in muscle weakness, hemolysis of red cells, coma and bone deformity, and impaired growth.

Low phosphate levels may be a sign of:

- Alcoholism
- Severe burns
- Diabetic ketoacidosis (too much acid in the blood)
- Overuse of diuretics (drugs that remove excess fluid from the body)
- Using too much antacid
- Hypercalcemia (too much calcium in blood)
- Hyperparathyroidism (high levels of the parathyroid hormone)
- Malnutrition
- Vitamin D deficiency conditions, such as rickets.

Alterations in Urinary Phosphate Concentration

Kidney problems can cause high or low levels of phosphate in the urine. Many medicines can change the results of this test, such as aspirin, corticoids, diuretics, parathyroid hormone or taking a lot of antacids.

High Urine Phosphate

High urine phosphate levels may be caused by:

- Kidney diseases.
- An overactive parathyroid gland (hyperparathyroidism).
- Too much vitamin D in the body.
- By eating a meal high in phosphorus.
- Some types of tumors

Low Urine Phosphate

Low urine phosphate levels may be caused by

- An underactive parathyroid gland (hypoparathyroidism).

- Kidney or liver diseases.
- Severe malnutrition.

The loss of too much phosphate into the urine may cause conditions that damage the bones, such as rickets or osteomalacia.

Body needs to maintain a delicate balance between levels of calcium and phosphorus. An abnormal result on a calcium test may indicate that phosphorus levels are also atypical.

Tests for calcium and creatinine levels may be done at the same time, as the phosphate urine test.

ESTIMATION OF SERUM AND URINARY INORGANIC PHOSPHATES

Estimation of Serum Inorganic Phosphorus

Principle

Phosphorus is estimated by **Fiske and Subbarow method** where the phosphate-containing solutions are treated with molybdic acid to produce phosphomolybdic acid, which is reduced by the addition of 1,2,4-aminonaphtholsulphonic acid, to give a blue colour. This is read at 660 nm.

Reagents

1. **TCA solution in water** (10%)
2. **H_2SO_4** (10 N and 5 N)
3. **H_2O_2** (30%) or fuming nitric acid
4. **Aminonaphthol-sulfonic acid reagent:** 195 mL of 15% sodium bisulfate solution is taken in a glass-stoppered cylinder and 0.5 g of 1,2,4-aminonaphtholsulfonic acid is added to it, followed by 5 mL of 20% sodium sulfite. It is stoppered and shaken until the powder is dissolved. If the solution is not complete, more of sodium sulfite, 1 mL at a time, is added with shaking. The solution is then transferred to a brown glass bottle and stored in the cold. This solution is usable for 4 weeks.
5. **Standard phosphate solution:** Weigh exactly 351 mg of anhydrous monopotassium phosphate (KH_2PO_4) is dissolved in water. Ten mL of 10 N H_2SO_4 is added and the solution is diluted to 1 L water, and mixed. This solution contains 0.4 mg of phosphorus in 5 mL. It is stable indefinitely.

Preparation of TCA Filtrate of Whole Blood

To 8 mL of a 10% solution, 2 mL of whole blood, serum or plasma is added slowly, with mixing. The mixture is shaken and filtered through a low ash filter paper.

Procedure

Two mL of TCA filtrate of serum (equivalent to 0.4 mL serum) is transferred to a test tube or micro-Kjeldahl flask and 2.5 mL of 5 N H_2SO_4 is added, also adding a quartz chip to minimize bumping. The contents of the flask are digested on a microburner. After evaporation is complete and the mixture turns brown or black with no further change, the tube is removed, cooled slightly and 1 drop of 30% H_2O_2 (if it gives too high a blank then add 1 or 2 drops of fuming nitric acid) is added to fall directly into the digestion mixture. The heating is then continued until the contents of the tube become colourless. When the mixture is clear, the tube is cooled, few mL of water is added, and the solution is heated to boiling, momentarily. The contents are then transferred to 25 mL volumetric flask quantitatively with washings. To the flask, is added 2.5 mL of 2.5% ammonium molybdate solution, followed by 1 mL of aminonaphtholsulphonic acid reagent. The solution is made up to 25 mL with water and mixed well. It is allowed to stand for 5 min, and the intensity of the colour is read in a colorimeter. The most accurate procedure is to run a digested blank standard and unknowns, simultaneously. The color of standard and unknown solutions is determined at 660 nm. The standard curve is plotted and the unknown solutions are read from the curve.

Calculation

$$\underset{\text{(mg/dL of serum)}}{\text{Total acid–soluble phosphorus}} = \text{mg P in unknown} \times \frac{100}{0.4}$$

Estimation of Urine Inorganic Phosphorus

Method is same as for serum. Dilute the urine 1:10 and calculate the value using the dilution factor.

If proteins are present in urine, addition of molybdate causes turbidity, so proteins should be removed. For this purpose, add 4 volumes of 10% TCA to 1 volume of urine, mix well and filter. Use an amount of filtrate between 0.5 and 1 mL, equivalent to 0.1 to 0.2 mL of urine, and proceed as above.

IRON AND TOTAL IRON-BINDING CAPACITY

Total quantity of iron (Fe) in a normal adult human body is about 4 g. 65% of the iron in the body is bound in hemoglobin molecules in red blood cells, about 4% in myoglobin molecules while around 30% is stored as ferritin or hemosiderin in the spleen, the bone and the liver. Small amounts of iron can be found in other molecules in cells throughout the body.

Normally, iron is present in most cells of the body in very small quantity, also in plasma and in extracellular fluid. Body conserves its iron supply so that less than 0.1% of the body iron content is lost daily, that too mostly in desquamated cells.

Total iron-binding capacity (**TIBC**), or sometimes also referred as **transferrin iron-binding capacity,** is a medical laboratory test that measures the blood's capacity to bind iron with transferrin. **Transferrin** is a nonheme iron protein involved in the transport of iron. It is a serum glycoprotein (β_1-globulin) synthesized in the liver as **apotransferrin** that has affinity for Fe^{3+}iron. Each apotransferritin molecule has two iron binding sites where iron is bound in the ferric form. After its binding with iron, apotransferrin is converted to transferrin. Thus, total iron binding capacity (TIBC) of plasma reflects serum transferrin concentration. Accordingly, it measures how well the transferrin proteins are transporting iron around the body.

Reference Range

- **Serum iron:** Men: 65–176 µg/dL
  Women: 50–170 µg/dL
- **TIBC:** 240–450 µg/dL

Reference value differs by as much as 35% between commercial methods. Many commercial methods tend to underestimate the true value of serum iron concentration by 25% and some are unreliable at concentration of serum iron less than 30 µg/dL. Methods which include deproteinization provide results that are substantially high than those results obtained by methods that do not include a step of deproteinization. So, the laboratory should define its own independent reference range.

Clinical Interpretation

Many medications can affect the results of a serum iron test by increasing or decreasing iron levels, e.g. birth control pills.

Serum iron concentration is decreased in

- Iron deficiency anemia
- Chronic inflammatory disorders
- Acute infections
- Immunization
- Acute or recent hemorrhage
- Heavy menstruation

Early symptoms of iron deficiency (anemia) include:

- Chronic fatigue
- Dizziness
- Headache
- Muscle weakness

Prolong deficiency may result in

- Difficulty in concentrating
- Grumpiness
- Tongue and mouth sores
- Pica (the compulsion to eat non-food items, such as paper or ice chips)
- Misshapen nails

Serum iron concentration is increased in:

- Hemochromatosis
- Aplastic anemia
- Iron poisoning in children
- After oral and parenteral iron medication

Symptoms of iron overload include:

- Pain in abdomen and joints
- Bronzing or darkening of skin
- Fatigue
- Heart problems
- Lack of energy
- Lack of sex drive
- Weight loss
- Muscle weakness

Abnormally high iron serum levels may mean one has consumed too much iron, vitamin B_6, or vitamin B_{12}. Serum iron levels in excess of 300 mg/dL (normal 50 to 150 mg/dL) are often associated with clinical symptoms. Levels in excess of 500 mg/dL are nearly always associated with clinical toxicity.

TIBC is raised in association with iron deficiency. In the non-anemic individual, the TIBC is approximately one-third saturated with iron. In pregnancy, there is a fall in the serum iron and percentage saturation of the TIBC.

TIBC is decreased in chronic inflammatory disorders or malignancies and in hemochromatosis.

ESTIMATION OF SERUM IRON AND TIBC

Serum Iron

- **Colorimetric method,** both **manual** and by **auto-analyzers**.
- **Atomic absorption spectrophotometry**

Principle

All protein-bound iron is released from transferrin by adding an acidic agent to lower the pH of the sample.

Following this, centrifugation permits the release of apotransferrin, iron-free transferrin and other plasma proteins. Ferric iron (Fe^{3+}) is reduced to the ferrous form (Fe^{2+}) in the supernatant. This is followed by the addition of a chromogenic iron-chelating agent, such as bathophenanthroline disulfonate or ferrozine, that complexes easily with ferrous iron. The resulting complex leads to color changes that are dependent on the concentration of the complex and the optical characteristics of the agent used. These changes are proportional to ambient iron concentrations.

TIBC

TIBC is determined by the addition of sufficient Fe^{3+} to saturate iron binding sites on transferrin. The excess iron is removed by adsorption with $MgCO_3$ powder and the assay for the iron content is then repeated. From this second measurement, TIBC is obtained.

Since serum iron concentration is to be measured, therefore, care should be taken to avoid using hemolyzed samples, which may falsely elevate iron concentrations.

MAGNESIUM

Adult human body contains nearly 20–25 g of magnesium. Nearly 75% of it is combined with calcium and phosphorus and is present in bones. Remaining of it is present in soft tissues, such as muscle and nervous tissue, and in body fluids.

In blood, most of the magnesium is intracellular. The concentration of magnesium in cells is 1–3 mMol/L. In general, higher the metabolic activity of the cell, higher the magnesium content. It is vital for energy production, muscle contraction, nerve function and the maintenance of strong bones. It is a cofactor for more than 300 enzymes involved in the metabolism of food components and the synthesis of many metabolic products. Among the reactions requiring magnesium are the synthesis of fatty acids and proteins, phosphorylation of glucose and its derivatives in the glycolytic pathway and transketolase reactions. Magnesium also plays a role in neuromuscular transmission.

Kidneys control magnesium balance by conserving magnesium efficiently, particularly, when intake is low. Low dietary intake of magnesium results in reduced urinary excretion of magnesium.

Normal Range

The normal range for serum magnesium is 1.7–2.3 mg/dL.

Clinical Interpretation

Hypomagnesemia

Hypomagnesemia, also spelled hypomagnesaemia, is an electrolyte disturbance in which there is a low level of magnesium in the blood.

Magnesium deficiency is often observed in alcoholics, patients on certain types of diuretics and in metabolic acidosis. Magnesium deficiency may also be observed in uremics as well as in children suffering from kwashiorkor. Low serum magnesium levels are also observed in pregnancy (particularly during toxemia of pregnancy), rickets, and diarrhea and vomiting.

Deficiency of magnesium can cause tiredness, generalized weakness, muscle cramps, abnormal heart rhythms, increased irritability of the nervous system with tremors, palpitations, hypokalemia, hypoparathyroidism which might result in hypocalcemia and tetany. Other symptoms that have been suggested to be associated with hypomagnesemia are confusion, disorientation, hallucinations, depression, hypertension and fast heart rate.

Hypermagnesemia

Increased blood levels of magnesium are seen in kidney failure, hyperparathyroidism, hypothyroidism, dehydration, diabetic acidosis, Addison's disease, parenteral treatment of pregnancy-induced hypertension and magnesium deficiency, lithium ingestion and use of magnesium-containing antacids or laxatives.

The signs and symptoms of an overdose include confusion, diarrhea, nausea, slow heart rate, upset stomach, vomiting and very low blood pressure. On rare occasions, magnesium overdose can lead to cardiac arrest or death.

ESTIMATION OF SERUM MAGNESIUM

Methods of Estimation

1. Photometric methods
2. Atomic absorption spectrophotometry
3. Flame emission
4. Fluorometric
5. Enzymatic methods (with hexokinase or other enzymes that use Mg^{2+}. ATP as a substrate).

Specimen Requirement

Serum and heparinized plasma are the preferred specimen for measuring Mg. Anticoagulants, like citrate, oxalate and EDTA, are not used as they form complex with Mg. Serum or plasma must be separated from clot or red blood cells as soon as possible, to prevent an increase in serum Mg because of its leakage from the cells, as RBCs are very rich in Mg. Thus, hemolyzed samples are unacceptable.

Urine specimen should be collected in acid, to prevent precipitation of Mg complexes.

Photometric Methods

Several metallochromic indicators or dye changes colour on selectively binding magnesium:

1. **Calmagite**, a metallochromic indicator, forms a coloured complex with Mg in alkaline solution which is measured at 530 nm.
   EDTA, a calcium chelating agent, is added to reduce Ca interference. Reagents may include potassium cyanide to prevent formation of heavy metal complexes, polyvinylpyrrolidone and surfactants to reduce interference from protein and lipemia.
2. **Methyl thymol blue** forms a blue complex with Mg, which is measured at 600 nm.
3. **Formazan dye** forms a complex with Mg in alkaline condition which is read at 600 nm by thin film reflectance photometry. There is little interference from icteric, hemolyzed or lipemic sample.
4. **Magon dye** also forms a red complex with Mg and is read at 600 nm.

Atomic Absorption Spectrophotometry

This method is more accurate and precise, and is used as a reference method for Mg. Mg is determined by atomic absorption spectrophotometry (AAS) after diluting the sample (1:50) with a solution of lanthanum.HCl, to eliminate interference from anions, including phosphate and proteins, and metal oxides. The specimen is aspirated into the air acetylene flame, in which the ground state Mg ions absorb light from a Mg hollow cathode lamp (285.2 nm). Absorption is directly proportional to the number of ground state magnesium atoms in the flame.

Free (Ionized Magnesium)

Free mg is measured by the use of ISE with neutral carrier ionophore. Estimation of free Mg may be helpful in critically ill patients, and during cardiopulmonary bypass, pre-eclampsia, neonatal distress, etc.

COPPER AND CERULOPLASMIN

Copper is essential to all living organisms as a trace dietary mineral. It is found mainly in the liver, muscle and bone. It is a key constituent of the respiratory enzyme complex, cytochrome C oxidase. Copper is also found in many superoxide dismutases, proteins that catalyze the decomposition of superoxide by converting it to oxygen and hydrogen peroxide. Copper also helps make melanin, bone and connective tissue.

The extent of intestinal copper absorption varies with dietary copper content and is about 50% at low copper intake and about 20% at high copper intake. Absorbed copper is transported to the liver in portal blood bound to albumin, where it is incorporated by the hepatocytes into cuproenzymes and other proteins and then exported in peripheral blood to various tissues and organs, in the form of ceruloplasmin.

A small amount of copper in plasma (<10%) is bound to albumin and is in equilibrium with plasma amino acids. This fraction may also be important for cellular uptake. Copper is excreted via bile into feces (0.5–2.0 mg/day). Small amounts of copper are also found in urine, sweat and menstrual blood.

Ceruloplasmin, also called ferroxidase I, is a copper-containing β_2-globulin present in plasma. It is synthesized in the liver in the form of apoprotein called apoceruloplasmin. After 6–8 atoms of copper are incorporated, apoceruloplasmin is converted to ceruloplasmin.

Ceruloplasmin helps in the conversion of Fe^{2+} to Fe^{3+} for its incorporation into transferrin and ferritin. It thus, helps in the transport of iron to extrahepatic tissues and acts as an enzyme at the erythrocyte-forming cells of the bone marrow. It also prevents membrane lipid oxidation under physiological conditions. At the same time, in the presence of superoxide (e.g. in inflamed vascular endothelium), ceruloplasmin is a major contributor to LDL oxidation.

Normal Value

- **Plasma copper:** 70–140 mg/dL. In adults, plasma copper below 50 mg/dL and for infants below 30 mg/dL, indicates probable copper depletion.
- **Urine copper excretion**: Normally, <60 mg/day and value above 200 mg/day is found in Wilson disease.
- **Serum ceruloplasmin:** 18–35 mg/dL.

Clinical Interpretation

- **The deficiency of copper** is characterized by anemia, neutropenia and skeletal abnormalities, especially, demineralization. Other changes may also develop, including subperiosteal hemorrhage, hair and skin depigmentation and defective elastin formation. The failure of erythropoiesis as well as cerebral and cerebellar degeneration, may lead to death.

- **Increased level of copper** can be responsible for disorders, such as hypotension, heart disease, premenstrual tension, postpartum depression, schizophrenia and childhood hyperactivity. Liver cirrhosis typically develops from toxic intake levels. Abnormalities in red blood cell formation also occur.
- **Hypercupriuria** is found in hemochromatosis, biliary cirrhosis, thyrotoxicosis, various infections and a variety of other acute, chronic and malignant diseases (including leukemia). Urine copper concentrations are also elevated in patients taking contraceptives or estrogens and during pregnancy.
- **Menkes syndrome,** also known as kinky-hair syndrome, is a sex-linked recessive defect that results in copper malabsorption, increased urinary copper loss and abnormal intracellular copper transport, all of which cause an abnormal distribution of copper among organs and within cells.
- **Low urine copper** levels are seen in malnutrition, hypoproteinemia, malabsorption and nephrotic syndrome. Increased zinc consumption interferes with normal copper absorption from the gastrointestinal tract causing hypocupremia.

Ceruloplasmin

It is a positive acute phase reactant and increases during infections and after tissue injury. Ceruloplasmin also increases during pregnancy and use of oral contraceptives, leading to a rise in serum copper concentration.

Wilson disease, also referred to as hepatolenticular degeneration, is characterized by excessive accumulation of copper in body tissues, such as the eyes, as a result of a genetic deficiency in the liver synthesis of **ceruloplasmin**.

ESTIMATION OF SERUM AND URINARY COPPER

Copper can be detected by

- Colorimetry
- Flame atomic absorption spectrophotometry (FAAS)

Flame atomic absorption spectrophotometry (FAAS) is more accurate. In the absorption technique, the sample is vaporized into free, neutral atoms and illuminated by a light source that emits the atomic spectrum of the element under analysis. The absorbance gives the quantitative measure of the concentration of the element.

Precautions should be taken against trace element contamination during collection, storage and processing of samples.

PRACTICAL EXERCISES

1. Estimation of calcium in serum
2. Estimation of calcium in urine
3. Estimation of phosphorus in serum
4. Estimation of phosphorus in urine
5. Estimation of serum iron and TIBC
6. Estimation of serum and urinary copper
7. Estimation of magnesium in serum
8. Estimation of ceruloplasmin in serum

Important Question

1. Write notes on:
    - i. Hypercalcemia
    - ii. Hypocalcemia
    - iii. Hypercalciuria
    - iv. Hypocalciuria
    - v. Hyperphosphatemia
    - vi. Hypophosphatemia
    - vii. TIBC
    - viii. Significance of serum iron
    - ix. Magnesium
    - x. Ceruloplasmin
    - xi. Menkes syndrome
    - xii. Wilson disease

Chapter

50

Hormone Profiles

Hormones are the body's chemical messengers and are part of the endocrine system. Endocrine glands make hormones, which travel through the bloodstream to tissues and organs. Hormones play a critical role in our body's chemistry, carrying messages between cells and organs. They affect our body's functions, from growth and sexual development, and mood to how well we sleep, how we manage stress and how our body breaks down food. But sometimes hormone levels are too high or too low. Hormone imbalances can occur any time regardless of one's age and cause serious health problems requiring ongoing medical management.

Hormone secretion occurs in response to specific biochemical signals from a wide range of regulatory systems. For instance, serum calcium concentration affects parathyroid hormone synthesis; blood glucose concentration affects insulin synthesis, etc. Regulation of hormone synthesis of gonadal hormones, adrenocortical hormones and thyroid hormones is often dependent on complex sets of direct influence and feedback interactions involving the hypothalamic-pituitary-adrenal (HPA), -gonadal (HPG), and -thyroid (HPT) axes.

Various hormones can be assayed by several techniques, which include

- Enzyme immunoassay or ELISA (*see* Chapter 39)
- Radioimmunoassay (RIA), and
- Chemiluminescence (*see* Chapter 41)
- Recently, a new immunoassay methodology has been applied to the measurement of some of the hormones, depending on the type of label attached to the antibody. The assay is variously called the **immunoradiometric assay**, the immunofluorimetric assay, the immunochemiluminometric assay or the immunoenzymometric assay.

Radioimmunoassay (RIA)

RIA is an important technique for estimation of hormones, steroids and drugs which are present in very minute quantities and cannot be detected by the colorimetric or other chemical methods.

Principle

The basic principle is the specificity of antigen (Ag) or antibody (Ab) reaction and is a competitive binding assay. Outline of various steps for the estimation of an antigenic analyte (immunogen), such as thyroid hormones, is as follows:

1. To generate antibodies against specific antigens, T_3 or T_4 is injected into rabbit.
2. These antibodies are purified and treated with radiolabeled antigen (T_3 or T_4 having ^{125}I) forming antigen–antibody (Ag–Ab) complex.
3. This Ag–Ab complex is then treated with patient's sample which has the unlabeled antigen.
4. Unlabeled antigen then displaces the labeled antigen, latter is then separated out and radioactivity is counted per minute, which is used to calculate unlabeled antigen in the patient's blood.

Reagents for RIA

1. Tracer (labeled antigen): The pure Ag is tagged with ^{125}I. Nonprotein antigens are labeled with tritium.
2. Antibodies against the test substance.
3. Standard which is the unlabeled antigen.
4. Buffer (pH 7–9).

Procedure

A series of test tubes with varying amount of pure Ag is incubated with fixed amount of labeled Ag (Ag*) and Ab for 6 hours. The free Ag is washed and radioactivity is counted in the bound (Ag*Ab) fraction. The test sample with unknown amount of Ag is processed similarly (Fig. 50.1).

A standard curve is prepared by taking known quantity of unlabeled Ag at different dilutions of standard. By referring to the standard curve, the amount of Ag can be obtained.

Methods of Separation of Bound and Unbound Antigen

1. Ion exchange chromatography.
2. Using second antibody against the first antibody.
3. Salt precipitation of Ag*Ab complex.
4. Immobilization of the Ab on a solid support and washing away of pure Ag.

Advantages of RIA

1. It can be used to assay immunogenic compounds available in pure form and can be radiolabeled.
2. It can also assay nonprotein compounds, e.g. hormones and drugs.
3. Sensitive and specific method.
4. It can be automated.

Disadvantages of RIA

1. Equipment and reagents are costly.
2. Half life of several radioisotopes is small, e.g. for ^{125}I (only 60 days), which requires frequent ordering.
3. Radiation hazards.
4. Lengthy procedure.
5. Trained personnel is required.

THYROID PROFILE

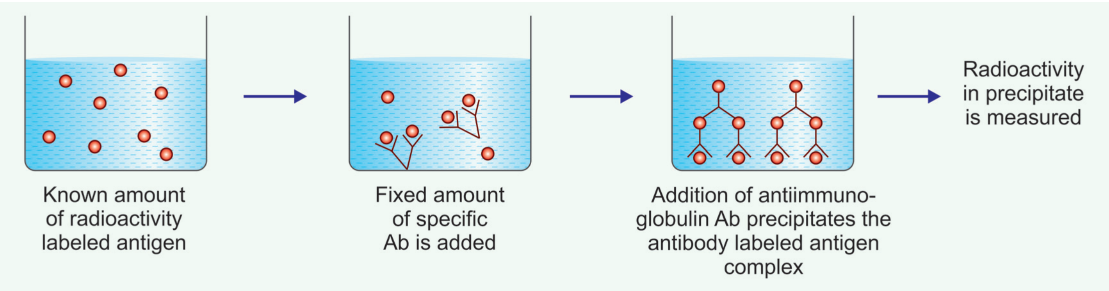
Thyroid profile, in general, includes estimations of thyroid hormones (T_3 and T_4) and the thyroid stimulating hormone (TSH).

THYROID HORMONES

The **thyroid hormones** are tyrosine-based hormones, produced by the thyroid gland that are primarily responsible for regulation of metabolism. The major form of thyroid hormone in the blood is thyroxine (T_4) and triiodothyronine (T_3 In humans, the ratio of T_4 to T_3 released into the blood is sometimes claimed to be quite high. T_4 is present in 50-folds excess in human plasma. It gets converted to T_3 (the metabolically active form) and is, thus, considered as a prohormone for T_3.

Only 0.2–0.4% of the total T_3 is present in the unbound form, also known as free T_3 (FT3). Rest is present in the bound form, i.e. bound to TBG, prealbumin and albumin. Similarly, FT4 is also a minute fraction of total T_4. Thyroxine and triiodothyronine can be measured as **free thyroxine** and **free triiodothyronine**, which are indicators of thyroxine and triiodothyronine activities in the body. They can also

Control


Known amount
of radioactivity
labeled antigen
Fixed amount
of specific
Ab is added
Addition of antiimmunoglobulin Ab precipitates the antibody labeled antigen complex
Radioactivity
in precipitate
is measured

Unknown Sample

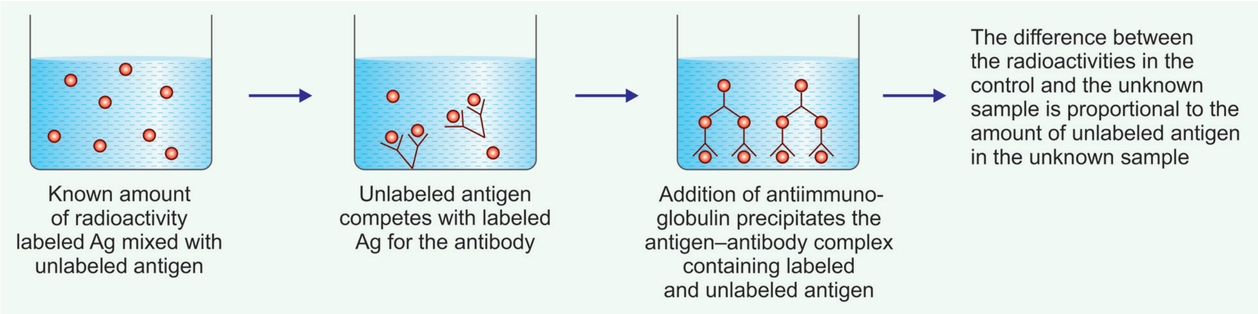

Known amount
of radioactivity
labeled Ag mixed with
unlabeled antigen
Unlabeled antigen
competes with labeled
Ag for the antibody
Addition of antiimmunoglobulin precipitates the antigen–antibody complex containing labeled and unlabeled antigen
The difference between the radioactivities in the control and the unknown sample is proportional to the amount of unlabeled antigen in the unknown sample

Fig. 50.1: General procedure for RIA

be measured as **total thyroxine** and **total triiodothyronine,** which also depend on the thyroxine and triiodothyronine that is bound to thyroxine-binding globulin.

Methods of Estimations of T_3 and T_4

For the estimation of serum thyroid hormones, RIA and ELISA are commonly used. For this purpose, serum is used and the fasting specimen is not essential. There is no interference from hemolyzed, icteric or lipemic samples in RIA. However, grossly hemolyzed, icteric or lipemic samples do interfere in ELISA.

Determination of T_3 by RIA

Principle

It is a solid phase RIA, where ^{125}I-labeled T_3 competes with T_3 in patient's serum for the specific antibody sites for a fixed time. This reaction takes place in the presence of blocking agents which liberate bound T_3 from the carrier proteins. After incubation, the supernatant is decanted and the antibody bound fraction of the radiolabeled T_3 can be measured using a gamma counter.

Procedure

RIA diagnostic kits are available which contain

- Total T_3 antibody coated tubes
- ^{125}I-labeled T_3

Six human serum calibrators for T_3 (A to F) with concentration ranging from 0 to 600 ng/dL.

Procedure is followed as per the kit insert and a standard curve is plotted, from which the patient's serum T_3 value is calculated.

Determination of T_4 by RIA

Principle

It is based on the same principle as for T_3 determination, except for the fact that ^{125}I-labeled T_4 is used. The antibody bound ^{125}I-T_4 can be measured by the gamma counter and is inversely related to patient's T_4 concentration.

Procedure

It is the same as for T_3 determination.

Determination of T_3/T_4 by ELISA

Principle

In this immunological reaction, enzyme along with the labeled antibodies are used.

- In the first step, serum T_3/T_4 and POD (peroxidase)-labeled T_3/T_4 compete with the limited quantity of T_3/T_4 specific antibodies coated onto the inside of test vial, respectively. The T_3/T_4-POD conjugate, not bound by the antibodies, is removed in the separation step.
- In the second incubation step, addition of hydrogen peroxide and a chromogen (ABTS: diammonium-2,2-azinobis-3-ethylbenzothiazoline-6-sulfonate) result in the formation of a colored complex, which can be measured at 405 nm. The absorbance is proportional to the enzyme activity of the antibody-T_3/T_4-POD complex bound to the wall of the vial. It is inversely related to T_3/T_4 concentration in the patient's serum.

Autoanalyzers

Determination of T_3, T_4 and TSH can also be done by using autoanalyzer.

THYROID-STIMULATING HORMONE

Thyroid-stimulating hormone (also known as **thyrotropin**, **thyrotropic hormone**, **TSH**, or **hTSH** for human TSH) is a pituitary hormone that stimulates the thyroid gland to produce T_4 and then T_3, which stimulates the metabolism of almost every tissue in the body.

Determination of TSH by IRMA

Principle

IRMA is a solid phase immunoradiometric assay, based on monoclonal and polyclonal anti-TSH antibodies, i.e. ^{125}I-labeled anti-TSH polyclonal antibody in liquid phase (say antibody 1) and monoclonal anti-TSH antibodies immobilized to the wall of a polystyrene tube (say antibody 2).

Procedure

- TSH (in patient's serum) is captured between antibody 2 and antibody 1.
- Unbound ^{125}I-labeled anti-TSH antibody is removed by decanting the tube.
- The TSH concentration is directly proportional to the radioactivity present in the tube, which is counted by the gamma counter. The concentration of TSH in the sample is obtained by the standard curve, made with the set of calibrators provided with the kit.

Reference Range

- T3: 86–187 ng/dL
- T4: 4.5–12.5 μg/dL
- TSH:0.3–5 μIU/mL

Clinical Interpretation

Both excess and deficiency of thyroxine can cause disorders. **Hyperthyroidism**, e.g. Graves' disease, is the clinical syndrome caused by an excess of circulating free thyroxine, free triiodothyronine, or both. **Hypothyroidism**, e.g. Hashimoto's thyroiditis, is the case, where there is a deficiency of thyroxine, triiodothyronine, or both.

- Primary hyperthyroidism: Elevated T_3, T_4 and decreased TSH.
- Primary hypothyroidism: Decreased T_3, T_4 and elevated TSH is seen.
- Secondary and tertiary hypothyroidism (decreased TSH and decreased TRH, respectively) is associated with low values of T_3 and T_4, due to pituitary and hypothalamic lesions. TSH is also suppressed to subnormal level.
- Increased level of TBG (thyroid binding globulin), like in pregnancy, hyperproteinemia or during estrogen therapy, is associated with elevated T_3 and T_4.
- Decreased level of TBG as in nephrotic, hepatic, hypoproteinemia, neoplastic disorders, acromegaly and in patients undergoing androgen or anabolic steroid therapy is associated with decreased level of T_3 and T_4.

Free T_3 (FT_3) and Free T_4 (FT_4)

Only 0.2–0.4% of the total T_3 is present in the unbound form known as free T_3 (FT_3). Rest is present in the bound form, bound to TBG, prealbumin and albumin. Similarly, FT_4 is also a minute fraction of total T_4. Nevertheless FT_3 and FT_4 are the biologically active fractions and can be determined accurately with the methods based on the principle of chemiluminescence.

INFERTILITY PROFILE

Infertility is the inability to get pregnant (conceive). It is typically diagnosed after a couple has tried to get pregnant for 12 months without success. Infertility may also be diagnosed when a woman has been able to get pregnant but has been unable to carry a fetus to term (miscarries). The infertility profile (female) is a test for important hormones, which can help build a picture of why a woman might not be conceiving. LH, FSH, estradiol and prolactin are the female sex hormones, which are commonly determined during the infertility workup in females along with testosterone, which is the male sex hormone.

FOLLICLE-STIMULATING HORMONE

Follicle-stimulating hormone (FSH) helps control a woman's menstrual cycle and the production of eggs. In men, it regulates the production and transportation of sperm. For women, FSH test is done on the third day of the menstrual cycle and is used to evaluate egg supply. For men, the test is used to determine sperm count.

ESTRADIOL

Estradiol is an important form of estrogen. An estradiol test is used to measure woman's ovarian function and to evaluate the quality of the eggs. Like FSH, it is done on the third day of a woman's menstrual cycle.

LUTEINIZING HORMONE

In women, luteinizing hormone (LH) is linked to ovarian hormone production and egg maturation. In men, it stimulates the hormone testosterone which affects sperm production. An LH test is used to measure a woman's ovarian reserve (egg supply) and a man's sperm count. It is done during a woman's menstrual cycle to see if she is ovulating.

PROGESTERONE

Progesterone is a female hormone produced by the ovaries during ovulation. It causes the endometrial lining of the uterus to get thicker, making it receptive for a fertilized egg. Serum progesterone test is used to determine if ovulation is occurring. Since progesterone levels increase towards the end of a woman's cycle, the test is done during the luteal phase of the menstrual cycle (just before her period starts).

PROLACTIN

The hormone prolactin is made by the pituitary gland and causes milk production. In women, a prolactin test is done to find out why they are not menstruating, or why they are having infertility problems or abnormal nipple discharge. The test is also done in men when there is a lack of sexual desire, difficulty getting an erection, or if there might be a problem with the pituitary gland.

TESTOSTERONE

Testosterone is the most well-known androgen that affects the sexual functioning of both men and women. In men, an androgen test is used to find the cause of a low sex drive, the inability to get an erection or infertility. In women, it is used to determine the cause of irregular periods or a low sex drive.

During the infertility workup in females, LH, FSH, estradiol and prolactin are the female sex hormones which are commonly determined with testosterone that is the male sex hormone. All of them control the making of sperm.

Various techniques are commonly used for their estimations. These include:

- RIAP
- ELISA
- Chemiluminescence

Reference Range

- LH (early follicular): 1.7–15 mIU/mL
- FSH (early follicular): 1.4–9.9 mIU/mL
- Prolactin: 2.8–28 ng/mL
- Testosterone: 14–76 ng/dL (females)
  260–1000 ng/dL (males)

Interpretation

- Increased serum testosterone and LH, and normal levels of FSH indicate PCOS (polycystic ovary syndrome) in females.
- Decreased levels of LH, FSH and testosterone in males indicate hypogonadism, which suggests a lesion at the level of hypothalamus or pituitary.
- Increased prolactin levels are seen in prolactinoma.
- Increased FSH level is seen in menopause, castration and seminiferous tubule failure.

PRACTICAL EXERCISES

1. Estimation of T_3, T_4 and TSH
2. Estimation of prolactin

Important Question

1. Write notes on:
   - i. Thyroid hormones
   - ii. Hyperthyroidism
   - iii. Hypothyroidism
   - iv. Infertility profile
   - v. RIA
   - vi. Thyroid profile

Chapter

51

Special Biochemical Investigations

TESTS FOR DIABETES

ESTIMATION OF BLOOD GLUCOSE

Estimation of blood glucose has been described in Chapter 23. It may include the:

- Random sample for blood glucose
- Fasting sample for blood glucose
- Two-hour's postprandial sample for blood glucose.

Besides, one can perform oral glucose tolerance test (OGTT).

GLUCOSE TOLERANCE TEST

Glucose tolerance test (GTT) determines the ability of an individual to tolerate an extra load of glucose.

Oral GTT is helpful in patients who have fasting blood glucose concentration in the upper normal limits, those with transient or sustained glycosuria without the symptoms of diabetes or those with family history of the disease, etc.

Procedure

- For a standard routine GTT fasting venous blood sample (after 12–16 hours of fast) is drawn, in a fluoride-oxalate bottle.
- Thereafter, 75 g of glucose (about 1 g/kg body weight) is given, in nearly 200–300 mL of water, to drink. Blood samples are again drawn after every 30 minutes, till 2 to 2½ hours.
- Urine specimens are also collected, simultaneously.
- Each sample is analyzed and glucose tolerance curve is plotted.

Interpretation of the Test

- Fasting blood glucose, maximum value of glucose, rate at which it returns to the fasting value and glycosuria gives idea about the disease.
- For a **normal individual fasting blood glucose** value is within the normal limit. It is increased by more than twofold within 60 min and thereafter returns to normal after about two hours of the ingestion of glucose. Moreover, glycosuria is not observed in any of the sample (Fig. 51.1).

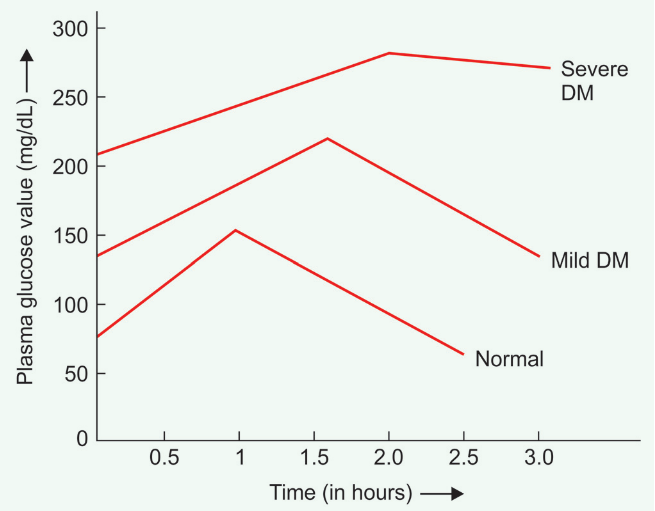


Fig. 51.1: Glucose tolerance curves

- Any deviation from the above is referred to as **abnormal glucose tolerance curve**. In a patient with diabetic mellitus fasting blood glucose level is above the normal and/or curve is raised as well as prolonged. This is also accompanied with glycosuria.
- In a patient of renal glycosuria, glucose tolerance curve is normal but there is glycosuria.

Fasting and Postprandial Blood Glucose Concentration

- Recently, International Committee of Diabetologists has recommended simplifying the glucose tolerance test to require only an overnight fasting measurement and 2 hours after a standard 75 g oral glucose load.

- An oral glucose tolerance test is normal if the fasting venous plasma glucose value is less than 100 mg/dL and the 2 hours value is below 140 mg/dL.
- A fasting value of 126 mg/dL or higher, or a 2-hour value of greater than 200 mg/dL is diagnostic of diabetes mellitus.
- Values between the upper limits of normal and those diagnostic of diabetes (fasting between 100 and 125 mg/dL and 2-hour value between 140 and 199 mg/dL) refers to impaired glucose tolerance (Table 51.1).

Table 51.1 Criteria for evaluating the fasting and 2 hours value

GTT	Fasting plasma glucose (mg/dL)	Two hours after 75 g glucose (mg/dL)
Normal	<100	<140
Impaired GT	100–125	≥140–199
Diabetes mellitus	≥126	≥200

Hypoglycemia (plasma glucose concentration below 40 mg/dL) is a common and potentially dangerous complication of insulin treatment, particularly, in tightly controlled diabetes. Insulinoma (benign adenoma of pancreatic islets) can also cause hypoglycemia. Estimating plasma insulin concentration, which is raised during fasting, can make its diagnosis.

Besides the above, **glycated hemoglobin** (glycosylated hemoglobin or HbA1c) is ordered for monitoring type II diabetes, while it becomes essential to test for **insulin**, if a patient is on insulin therapy.

GLYCATED HEMOGLOBIN

Glycated hemoglobin (HbA1c) reflects the average blood sugar concentration for a time period of 6–8 weeks and remains unaffected by the short-term fluctuations in blood sugar. Compared with repeated daily blood sugar test, HbA1c helps in evaluating the adequacy of diabetic control. An elevated HbA1c level may signal the need for a change in insulin regimen or meal plan. However, target HbA1c goal may vary depending on age and various other factors.

HbA1c can be measured by

- Ion exchange method
- HPLC

Ion Exchange Method

Commercially available kits are used for this purpose. Hemolysate is prepared by using whole blood, which is then mixed with a weakly binding cation exchange resin. The nonglycated hemoglobin binds to the resin leaving glycated Hb (GHb) in the supernatant. The GHb % is determined by measuring the absorbance of the GHb fraction and that of the total Hb.

HPLC

This is a very precise method where whole blood sample is used. The sample is first treated with hemolyzing reagent and then applied to the cation exchange column, where hemoglobin is separated, based on the attraction of hemoglobin to the column. The separated hemoglobin (HbA1c) then passes through the flow cell of the filter photometer, where changes in absorbance at 415 nm are measured. The background variations are corrected by an additional filter at 690 nm. A chromatogram of the changes in absorbance versus the retention time is obtained.

Reference Range

4–6.0%.

Clinical Interpretation

- **High levels** are directly linked to the microvascular complications, like retinopathy and nephropathy. High HbA1c are also seen in iron deficiency anemia, probably because of high proportion of old erythrocytes.
- **Decreased levels** may be seen in hemolytic disease or in other conditions with shortened red cell survival.

INSULIN

People with type 1 diabetes need insulin therapy to survive. Many people with type 2 diabetes or gestational diabetes also need insulin therapy. Often insulin is injected using a fine needle and syringe or an insulin pen, a device that looks like a large ink pen. An insulin pump may also be an option.

Insulin can be estimated, very accurately, by chemiluminescence as well as ELISA.

Reference Range

2–25 μIU/mL.

Clinical Interpretation

Insulin determination is of value in identifying those diabetic patients who would require insulin treatment. Individuals with a peak plasma insulin levels exceeding 60 μIU/mL after a 75 g oral glucose, are unlikely to develop microvascular complications and can be controlled by diet. Those patients with peak insulin concentration less than 40 μIU/mL require insulin treatment and are more prone for microvascular complications.

Insulin determination is also useful in evaluation of fasting hypoglycemia.

Measurement of **C-peptide** is a better indicator of β cell function. C-peptide assay does not measure exogenous insulin. It also does not cross react with insulin antibodies, which interfere with insulin immunoassay.

CARDIAC MARKERS

Cardiac markers are biomarkers, which are measured to evaluate heart function. They are often discussed in the context of myocardial infarction, but other conditions can also lead to an elevation in cardiac markers levels. Most of the early markers identified include enzymes, e.g. CPK and its isoenzyme CPK-MB, LDH and AST (*see* Chapter 35). Besides, troponin (troponins I and T), though not enzyme, is also an important cardiac marker.

CREATINE PHOSPHOKINASE

Creatine phosphokinase (CPK) or creatine kinase (CK), also known as phosphocreatine kinase, is an enzyme expressed by various tissues and cell types. CPK catalyses the conversion of creatine and utilizes ATP to form phosphocreatine and ADP. This reaction is reversible and, thus, ATP can be generated from phosphocreatine and ADP.

In tissues and cells that consume ATP rapidly, especially skeletal muscle and smooth muscle, phosphocreatine serves as an energy reservoir for the rapid buffering and regeneration of ATP *in situ*, as well as for intracellular energy transport by the phosphocreatine shuttle. Thus, creatine kinase is an important enzyme in such tissues.

Clinically, creatine kinase is assayed in blood tests as a marker of damage of CK-rich tissues, such as in myocardial infarction and muscular dystrophy. Increased levels are also seen in polymyositis, motor neuron disease and in acute cerebrovascular accidents.

Creatine kinase has 3 isoenzymes, i.e. CPK-BB (CK-1, brain), CPK-MB (CK-2, heart) and CK-MM (CK-3, skeletal muscle).

Determination of CPK by Colorimetric Method

Principle

CPK catalyses the following reaction:

Creatine phosphate + ADP ↔ Creatine + ATP

Creatine so formed reacts with diacetyl and α-naphthol in alkaline medium to give a colored complex, which is read at 520 nm.

Reagents

1. **Creatine phosphate:** Freshly prepared, by mixing 3 mg of creatine phosphate in 1 mL distilled water (DW). Add 0.4 mL of reagent 3 and 2 drops of reagent 4. Mix them properly. Add 24 mg of cysteine hydrochloride and mix thoroughly.
2. **ADP:** Freshly prepared, by dissolving 0.75 mg of ADP in 0.3 mL DW.
3. **Tris buffer (100 mM/L; pH 7.4):** 12.1 g of Tris in 820 mL of 0.1 N HCl. Final volume is made to 1 L with DW.
4. **Sodium hydroxide:** 0.1 N.
5. **p-chloromercuribenzoic acid:** 1.07 g/dL in 25 mL of 1 N sodium hydroxide, 22 mL of 1 N HCl and 53 mL of DW.
6. **Alkaline EDTA:** Dissolve 1 g of disodium salt of ethylenediamine tetra-acetic acid and 10 mL of 1 N sodium hydroxide in 1 L of DW.
7. **Sodium hydroxide:** 3 N
8. **Sodium carbonate:** 6 N
9. **α-Naphthol:** Freshly prepared, by mixing 50 mg of α-naphthol in 1.5 mL of reagent 8.
10. **Diacetyl (stock solution):** 1 g/dL in DW. Dilute this reagent 1:20 before use, to make it working diacetyl reagent.
11. **Working standard:** 29.8 mg (2 mmol/mL) creatine monohydrate in 100 mL of reagent 6.

Procedure

Take 3 test tubes and mark them as test (T), standard (S) and blank (B). Add various reagents and solutions as shown in Table 51.2.

Table 51.2 Preparation of test tubes for the estimation of total CPK in serum by colorimetric method

	T	S	B
Substrate, mL	0.4	0.4	0.4
DW, mL	–	0.2	0.2
Serum, mL	0.05	–	0.05
Reagent 2, mL	0.2	–	–
Mix well and incubate at 37°C for 30 min.			
Working std, mL	–	0.05	–
Reagent 5, mL	0.5	0.5	0.5
Reagent 6, mL	3.75	3.75	3.75
Reagent 9, mL	1.0	1.0	1.0
Reagent 10, mL	0.5	0.5	0.5

Mix well and keep the tubes in dark at room temperature for 30 min. Measure OD against distilled water at 520 nm.

Calculation

$$\text{Serum CPK activity, IU} = \frac{\text{OD test} - \text{OD blank}}{\text{OD std} - \text{OD blank}} \times 66.7$$

Reference Range

- Men: 20–50 IU
- Women: 10–37 IU

CPK-MB

As mentioned above, creatine kinase has 3 isoenzymes, out of which CPK-MB (CK-2) is predominant in cardiac muscle. Its activity is determined by **immuno-inhibition method**.

Principle

The patient's specimen is treated with the reagent containing polyclonal antibody to CK-M monomer, which completely inhibits the activity of CK-MM and one-half activity of CK-MB.

As the activity of CK-BB in serum is almost zero, the activity of the uninhibited B monomer subunit of CK-MB is measured, which represents half the activity of CK-MB. The activity of CK-MB is measured by UV-kinetic method, using creatine phosphokinase (CPK), hexokinase (HK) and glucose-6-phosphate dehydrogenase (G6PD) enzymes, based on the following reactions:

$$\text{Creatine phosphate} + \text{ADP} \xrightarrow{\text{CPK}} \text{Creatine} + \text{ADP}$$

$$\text{Glucose} + \text{ATP} \xrightarrow{\text{HK}} \text{Glucose-6-P} + \text{ADP}$$

$$\text{Glucose-6-P} + \text{NADP} \xrightarrow{\text{G6PD}} \text{6-Phosphogluconate} + \text{NADPH} + \text{H}^+$$

Procedure

- Substrate: 0.5 mL
- Serum: 20 µL

Mix and read the initial absorbance at 340 nm, after the lag phase of 600 sec. Repeat readings after a kinetic interval of 300 sec.

CPK-MB (IU) = 1651 × ΔA/min (37°C)

Reference Range

0–24 IU.

Interpretation

An increase in **CPK-MB** activity is seen within 6 hours following a myocardial infarction. The value peaks in about 24 hours and returns to normal in about 2–3 days. The area under the peak and the slope of initial rise is proportional to the size of infarct. In such patients CPK(MB) isoenzyme, usually exceeds 6% of the total activity. In patients of MI, though, first rise is seen within 6 hours after the onset of symptoms but for diagnosis with high specificity and sensitivity serial sampling over a period of 8–12 hours is required.

LACTATE DEHYDROGENASE

Lactate dehydrogenase (**LDH** or **LD**) is an enzyme involved in energy production that is found in almost all the body's cells, with the highest levels found in the cells of the heart, liver, muscles, kidneys, lungs and blood cells. LDH catalyzes the conversion of lactate to pyruvic acid and back, as it converts NAD^+ to NADH and back.

Determination of LDH is generally, done by **UV kinetic method**, for which kits are available where the instructions of adding the substrate is mentioned.

Principle

LDH catalyses the following reaction:

$$\text{L-lactate} + \text{NAD}^+ \leftrightarrow \text{Pyruvate} + \text{NADH} + \text{H}^+$$

Increase in OD is measured after 45 sec, by the interval of 1 min at 340 nm. Determine the mean absorbency change per minute (ΔA/min).

If the absorbance change exceeds 0.100 at 340 nm, then dilute the serum 1:10 by using normal saline (result × 10)

Calculation

LDH, IU = 9807 × ΔA/min

Reference Range

70–240 IU.

Interpretation

Elevated levels of LDH in blood, usually, indicate some type of tissue damage. LDH levels will rise as the cellular destruction begins, peak after some time period, and then begin to fall. LDH levels are elevated in a wide variety of conditions, reflecting its widespread tissue distribution.

An elevated level of LDH may be seen in patients with

- Hemolytic anemia
- Pernicious anemia (megaloblastic anemia)
- Infections, such as infectious mononucleosis, meningitis, encephalitis and HIV.

- Sepsis
- Intestinal and lung (pulmonary) infarction
- Acute kidney disease
- Acute liver disease
- Acute muscle injury
- Pancreatitis
- Bone fractures
- Testicular cancer, lymphoma, or other cancers

Low levels are sometimes seen when someone ingests large amount of ascorbic acid (vitamin C).

TROPONINS

Troponin or the **troponin complex** is a component of thin filaments (along with actin and tropomyosin) and is the protein complex to which calcium binds to trigger the production of muscular force. It is integral to muscle contraction in skeletal muscle and cardiac muscle but not smooth muscle.

Troponin itself has three subunits, i.e. troponin C (TnC), troponin I (TnI) and troponin T (TnT), each playing a role in force regulation.

Individual subunits serve different functions

- TnT is a tropomyosin-binding subunit. It regulates the interaction of troponin complex with thin filaments, interlocking them to form a troponin-tropomyosin complex.
- TnI inhibits ATPase activity of actomyosin. It binds to actin in thin myofilaments to hold the troponin-tropomyosin complex in place.
- TnC is a Ca^{2+}-binding subunit. It binds to calcium ions to produce a conformational change in TnI, thereby, playing the main role in Ca^{2+} dependent regulation of muscle contraction.

Troponin I and troponin T are found in heart muscle and are released into the blood when there is damage to the heart. So, troponin tests are primarily ordered to help diagnose a heart attack and rule out other conditions with similar signs and symptoms.

Troponin tests are sometimes ordered along with other cardiac markers, such as CK-MB or myoglobin. However, troponin is the preferred test for a suspected heart attack because it is more specific for heart injury than other tests (which may be elevated in the blood with skeletal muscle injury) and remain elevated for a longer period of time.

Either a troponin I or troponin T test can be performed. The concentrations are different but they basically provide the same information.

Determination of Troponin T (Rapid Test)

This is a qualitative immunological test. Only heparinized venous whole blood should be used.

Principle

The test application area contains two monoclonal antibodies specific for TnT. One is gold labeled and other is biotinylated. If TnT is present in the specimen, it forms a sandwich complex with two antibodies and appears as a red streak at the test zone. Excess gold labeled antibodies gather along the control line and give a red streak. So, two red lines mean a positive test and one red line denotes a negative test. If control line is absent, result must not be used.

TnI can be measured by chemiluminescence with high accuracy.

Reference Range

TnI 0–1.5 ng/mL.

Interpretation

A troponin test measures the levels of troponin T or troponin I proteins in the blood. These proteins are released when the heart muscle has been damaged, such as occurs with a heart attack. The more damage there is to the heart, the greater the amount of troponin T and I there will be in the blood. So, serial sampling must be done.

Both proteins are now widely used to diagnose acute myocardial infarction (AMI), unstable angina, post-surgery myocardium trauma and some other diseases related with cardiac muscle injury.

They can be detected in patient's blood 3–6 hours after onset of the chest pain, reaching peak level within 16–30 hours. Elevated concentration of TnI and TnT in blood samples can be detected even 5–8 days after onset of the symptoms, making both proteins useful for the late diagnosis of AMI.

Troponin tests are also sometimes used to evaluate people for heart injury due to causes other than a heart attack or to distinguish signs and symptoms, such as chest pain that may be due to other causes. Testing may also be done to evaluate people with angina, if their signs and symptoms worsen.

Myocardial injury can be better assessed by the levels of troponin T and I, as they are specific for myocardial injury unlike CPK, which increases in muscular injuries as well.

ENZYME MARKERS FOR A LIVER DISEASE

Estimation of the following enzymes helps in the diagnosis of a liver disease:

- SGPT/SGOT (*see* Chapter 35)
- Alkaline phosphatase (*see* Chapter 35)
- Gamma-glutamyltransferase (γ-GT)

GAMMA-GLUTAMYLTRANSFERASE

Gamma-glutamyltransferase (γ-GT or GGT) is an enzyme that catalyses the transfer of γ-glutamyl group from peptides and compounds that contain it to some acceptor. The acceptor is the substrate itself, some amino acid or peptide, or even water in which case simple hydrolysis takes place. Glycylglycine is five times more effective as an acceptor than either glycine or the tripeptide. GGT is present in proximal renal tubule, liver, pancreas and intestine. GGT activity in serum comes primarily from liver. It is a sensitive indicator of the presence of hepatobiliary disease.

Determination of GGT Activity

Principle

In IFCC reference measurement procedure for GGT, L-γ-glutamyl-3-carboxy-4-nitroanilide serves as a substrate, with glycylglycine serving as an acceptor. Buffering is provided by glycylglycine itself. Temperature is 37°C and the product so formed, 5-amino-2-nitrobenzoate, is measured at 410 nm.

GGT is a stable enzyme *in vitro*. Activity is stable for 1 month at 4°C and for 1 year at –20°C.

Reference Value

Adults: 5–35 U/L.

Interpretation

In normal full-term neonates, GGT activity at birth is approximately six to seven times the adult reference range. The activity then declines and reaches adult value by the age of 5–7 months.

- High elevations in primary and metastatic liver neoplasms
- Moderate elevation (2–5 times the normal): infectious hepatitis
- Small increase: Fatty liver, heavy drinkers, patients on anticonvulsant drugs.

PRACTICAL EXERCISES

1. Estimation of glycated hemoglobin
2. Estimation of serum insulin
3. Estimation of troponin
4. Estimation of CK-MB
5. Estimation of LDH
6. Estimation of serum γ-GT

Important Question

1. Write notes on:
    i. Cardiac markers
    ii. Glycated hemoglobin
    iii. Insulin
    iv. Troponins
    v. Reference range and diagnostic significance of CK-MB
    vi. Reference range and diagnostic significance of LDH
    vii. Reference range and diagnostic significance of γ-GT

Chapter

52

Biochemical Analysis of CSF

Cerebrospinal fluid (CSF) circulates around the brain and spinal column, and is secreted by the choroid plexuses, around the cerebral vessels and along the wall of the ventricles of the brain. More than 80% of the CSF protein content originates from the plasma by ultrafiltration and pinocytosis, and the remainder from intrathecal synthesis. The lowest concentration of total protein and the smallest proportion of the larger protein molecules are in ventricular fluid. As the CSF passes down to the lumbar spine, the protein concentration increases. It helps in the diagnosis of a variety of disorders, particularly **meningitis**.

MENINGITIS

Meningitis is an acute inflammation of the protective membranes covering the brain and spinal cord, collectively known as the meninges. It may be caused by infection with viruses, bacteria or other microorganisms and less commonly by certain drugs.

Viral meningitis

It is caused by enteroviruses, herpes simplex virus, varicella zoster virus, mumps virus, HIV, etc.

Bacterial meningitis

It can be caused by several strains of bacteria, most commonly which include *Streptococcus pneumoniae* (pneumococcus), *Neisseria meningitidis* (meningococcus), *Haemophilus influenzae* (Haemophilus) and *Listeria monocytogenes* (Listeria).

Tuberculous meningitis

It is caused by *Mycobacterium tuberculosis*. It is more common in people from countries in which tuberculosis is endemic, but is also encountered in persons with immune problems, such as AIDS.

Fungal meningitis

It is relatively uncommon and causes chronic meningitis.

For the diagnosis of the cause, CSF is normally obtained from the lumbar spinal region. Biochemically, CSF is tested for glucose, protein and chloride.

Reference Range

Reference range of normal spinal fluid is given below:

- Proteins: 20–40 mg/dL
- Glucose: 50–75 mg/dL
- Chloride: 118–132 mmol/L

ESTIMATION OF PROTEIN IN CSF

Since CSF is an ultrafiltrate of plasma, low molecular weight plasma proteins, like prealbumin, albumin and transferrin, predominate. Total proteins in CSF can be estimated by:

1. **Turbidimetric method:** Light scattering occurs when radiant energy passes through a solution in which the molecules are in collision. Turbidity decreases the intensity of incident beam of light as it passes through the solution of particles. The measurement of this decrease in intensity is called turbidimetry. It is measured at 180° from the incident beam on spectrophotometer and clinical analyzers. It requires 0.2–0.5 mL of the sample.
2. **Dye binding method** (Coomassie brilliant blue, CBB): The method is simple and fast. CBB binds to protonated amine group of amino acid residues in a polypeptide chain, resulting in decreased absorbance at 465 nm and increased absorbance at 595 nm. Sample volume required is 25 µL.
3. **Nephelometry, immunoturbidimetry, electroimmunodiffusion** and **mass spectrometry** are most often used for measurement of albumin and IgG in CSF.

Interpretation

The protein concentration in CSF varies with age and level of tapping (e.g. lumbar, ventricular, etc). It correlates well with the concentration of total proteins and different fractions in serum, but they are significantly low.

The predominant fraction in CSF is albumin, similarly as in serum.

- **Decreased total protein concentration in CSF** is generally associated with CSF leakage.
- **Increased protein level** may be a sign of a tumor, bleeding, nerve inflammation, or injury. A blockage in the flow of spinal fluid can cause the rapid buildup of protein in the lower spinal area.

Range

- Bacterial meningitis: 80–500 mg/dL
- Viral meningitis: 30–100 mg/dL
- Coccal meningitis: 100–500 mg/dL
- TB meningitis: 50–300 mg/dL (up to 1000 mg/dL)

ESTIMATION OF GLUCOSE IN CSF

Glucose in CSF can be estimated by hexokinase, GOD-POD, glucose dehydrogenase methods, as for serum/plasma. CSF should be analyzed for glucose immediately, as it may be contaminated with bacteria and other cells.

Further, CSF glucose must always be compared with concurrently measured plasma glucose for adequate clinical interpretation.

Interpretation

CSF: serum glucose ratio is approximately 0.6 in healthy adults (ventricular CSF glucose is higher than lumbar by 6–18 mg/dL). Normal concentration of glucose in CSF is 60–80% of that in the plasma (for glucose plasma concentration less than 400 mg/dL).

- **Normal CSF glucose concentration** is observed in viral CNS infections with the exceptions of mumps, enterovirus, lymphocytic choriomeningitis, herpes simplex and herpes zoster virus.
- **CSF glucose is low** in bacterial, mycobacterial, fungal and malignant process invading meninges, subarachnoid hemorrhage and CNS sarcoidosis. Concentration less than 18 mg/dL is strongly predictive of bacterial infection.
- **CSF glucose rarely exceeds** 288 mg/dL even in patients with severe hyperglycemia.

Alterations in Glucose and Protein Levels in Different Types of Meningitis

Aseptic (Viral) Meningitis

- Glucose level: >60% serum glucose (may be low in HIV infection)
- Protein level: Elevated (>50 mg/dL)

Bacterial Meningitis

- Glucose level: Low (<40% of serum glucose)
- Protein level: Elevated (>50 mg/dL)

Tubercular Meningitis

- Glucose level: Low
- Protein level: Elevated

ESTIMATION OF CHLORIDE IN CSF

Due to the way it is produced, CSF has a higher chloride level than plasma, and an equivalent sodium level. The chloride contents in CSF and plasma, and the ratio of CSF chloride to that of plasma has no diagnostic significance for viral meningitis. The chloride content in CSF is significantly lowered in tuberculous meningitis. However, CSF and blood Cl^- levels and the blood/CSF Cl^- ratio are not useful in differentiating between TBM, acute bacterial meningitis and viral meningitis.

PRACTICAL EXERCISE

1. Estimate glucose, protein and chloride in CSF.

Important Question

1. Write notes on:
   - i. Meningitis
   - ii. Diagnostic significance of biochemical tests in CSF

Chapter 53

Biochemical Analysis of Ascitic and Pleural Fluids

ASCITIC FLUID

The organs of the abdomen are contained in a sac or membrane called the **peritoneum**. Normally, the peritoneal cavity contains only a small amount of fluid. Abnormal accumulation of **ascitic fluid** (protein-containing fluid) within the abdominal (peritoneal) cavity is referred to as **ascites**. Technically, it is more than 25 mL of fluid in the peritoneal cavity.

Causes of Ascites

Most commonly, ascites is due to liver disease and the inability of that organ to produce enough protein to retain fluid in the bloodstream as well as obstruction to flow through the scarred cirrhotic liver. Amounts up to 35 liters are possible.

Causes of Ascites due to Liver Problems

- **Cirrhosis** describes a form of liver disease in which liver tissue, that is damaged, is replaced by scar tissue. Alcoholic liver disease or alcoholic hepatitis (inflammation), viral hepatitis (B or C) and fatty liver disease are the most common causes for cirrhosis.
- **Acute liver failure** may be due to any acute injury to liver cells including adverse reactions to medications or drug abuse.
- **Cancer** that has metastasized or spread to the liver can also be the source of ascites.

Other Causes

- **Heart failure,** the inability of the heart muscle to adequately pump the fluid within the blood vessels.
- **Nephrotic syndrome**, in which kidney damage causes protein to leak into the urine, decreases oncotic pressure and may result in ascites.
- **Disorders of the pancreas:**
  - **Acute pancreatitis:** Inflammation of the pancreas.
  - Chronic pancreatitis can result in malnutrition, leading to decreased total body protein, loss of oncotic pressure, and ascites.
  - Pancreatic cancer can cause direct fluid loss.
- **Direct irritation of the peritoneum** may be due to a malignancy (cancer) or infection.
- **Diseases of the ovary**
- **Hypothyroidism**

BIOCHEMICAL TESTS

Ascitic fluid can accumulate as a **transudate** or an **exudate**. Roughly **transudates** are a result of increased pressure in the hepatic portal vein (>8 mmHg, usually around 20 mmHg), e.g. due to cirrhosis, while **exudates** are actively secreted fluid due to inflammation or malignancy.

Exudates

Exudates are high in protein and lactate dehydrogenase (LDH), and have a low pH (<7.30), a low glucose level and more white blood cells.

Transudates

Transudates have low protein (<30 g/L), low LDH, high pH, normal glucose and fewer than 1 white cell per 1000 mm^3.

Laboratory tests on the ascitic fluid may include evaluation for infection and the assessment of electrolytes and other substances, i.e.:

- **Total proteins:** In the past, ascitic fluid has been classified as an exudate if the protein level is greater than or equal to 2.5 g/dL. However, the accuracy is only approximately 56% for detecting exudative causes.

- **The serum-ascites albumin gradient (SAAG):** The most useful measure is the difference between ascitic fluid and serum albumin concentrations. Calculated by subtracting the ascitic fluid albumin value from the serum albumin value, it correlates directly with portal pressure.
  A high gradient (> 1.1 g/dL) indicates that the **ascites is due to portal hypertension**. **A low gradient** (< 1.1 g/dL) indicates **ascites of nonportal hypertensive** as a cause.
  The specimens for this purpose should be obtained relatively, simultaneously. The accuracy of the SAAG results is approximately 97% in classifying ascites.
  The total protein level may provide additional clues when used with the SAAG. An **elevated SAAG and a high protein level** are observed in most cases of ascites due to **hepatic congestion**. The combination of a **low SAAG and a high protein level** is characteristic of **malignant ascites**.
  Thus, the terms high-albumin gradient and low-albumin gradient have replaced the terms transudative and exudative in the description of ascites.
- **Ascitic amylase** cultures/**PCR** are recommended for suspected pancreatitis while mycobacterial cultures/PCR for suspected tuberculosis.

PLEURAL FLUID

Thin membranes, called **pleura**, line both the lungs and chest cavity in the body. In healthy lungs, these membranes keep a small amount of liquid between the lungs and chest to prevent friction as the lungs expand and contract during breathing.

Pleural fluid is the fluid that is found between the layers of the pleura, the membranes of which line the cavity and surround the lungs, called the **pleural space**. A **pleural effusion** is excess fluid that accumulates in the pleural cavity, which can impair breathing by limiting the expansion of the lungs. Pleural effusion is always a secondary disorder, meaning that it is caused by something else.

Causes of Pleural Effusion

Some of the most common causes of pleural effusion are other diseases and disorders, including:

- **Liver or kidney disease:** Diseases such as cirrhosis may cause fluid to build-up in the body and leak into the chest.
- **Congestive heart failure:** Failure of the heart to pump blood properly can cause a build-up of liquid in the chest.
- **Autoimmune disorders:** Disorders such as lupus or rheumatoid arthritis may target the lungs and create problems with the pleura.
- **Infections:** Respiratory infections such as tuberculosis and pneumonia could progress and cause accumulation of water in the lungs.

Types of Effusions

One of the most important steps in diagnosis is determining the type of effusion, which depends on whether the process producing the effusion is local (exudate) or systemic (transudate).

Transudate Effusion

A transudate effusion is usually caused by fluid leaking back into the chest due to other disorders that cause a pressure imbalance in the blood vessels. The liquid in the effusion is typically made up of compounds found in the blood plasma and contains little or no protein, and low levels of cholesterol. The most common causes of transudative pleural effusions are congestive heart failure and liver cirrhosis. Other causes include severe hypoalbuminemia, nephrotic syndrome, myxedema, peritoneal dialysis and end-stage kidney disease.

Exudate Effusion

Exudate effusions are caused by blocked blood or lymph vessels in the area. Exudate is cloudy in appearance, and it normally contains high levels of protein and cholesterol. The most common causes of exudative pleural effusions are bacterial pneumonia, cancer, viral infection and pulmonary embolism.

When a pleural effusion has been determined to be exudative, additional evaluation may be done to determine its cause. For this purpose, **amylase**, **glucose**, **pH** and **cell counts** are measured.

- Red blood cell counts are elevated in cases of bloody effusions (for example, after heart surgery or hemothorax from incomplete evacuation of blood).
- **Amylase** levels are elevated in cases of esophageal rupture, pancreatic pleural effusion, or cancer.
- **Glucose** is decreased with cancer, bacterial infections, or rheumatoid pleuritis.
- **pH** is low in emphysema (<7.2) and may be low in cancer.
- If tuberculosis is the cause, examination for *Mycobacterium tuberculosis* should be done. PCR for tuberculous DNA or adenosine deaminase levels may also be checked.

Further, an accurate diagnosis of the cause of the effusion, transudate versus exudate, relies on a comparison of the chemistries in the pleural fluid to those in the blood, using Light's criteria. According to Light's criteria, a pleural effusion is likely exudative, if at least one of the following exists:

1. The ratio of pleural fluid protein to serum protein is greater than 0.5.
2. The ratio of pleural fluid LDH and serum LDH is greater than 0.6.
3. The difference between the albumin level in the blood and the pleural fluid greater than 1.2 g/dL suggests a transudative pleural effusion.

PRACTICAL EXERCISES

1. Estimation of total protein and glucose in pleural fluid
2. Estimation of total proteins in ascitic fluid

Important Questions

1. Differentiate between transudate and exudate.
2. Write notes on:
   i. Significance of biochemical tests in ascitic fluid
   ii. Significance of biochemical test in pleural fluid

Chapter

54

Biochemical Examination of Stool

A stool test involves the collection and analysis of fecal matter to diagnose the presence or absence of a medical condition.

COMPOSITION OF STOOL

- Normally, feces are made up of 75% water and 25% solid matter. About 30% of the solid matter consists of dead bacteria; about 30% consists of indigestible food matter such as cellulose; 10 to 20% is cholesterol and other fats; 10 to 20% is inorganic substances, such as calcium phosphate and iron phosphate; and 2 to 3% is protein. Cell debris, shed from the mucous membrane of the intestinal tract, also passes in the waste material, as do bile pigments (bilirubin) and dead leukocytes (white blood cells).
- The brown color of feces is due to the action of bacteria on bilirubin, which is the end product of the breakdown of hemoglobin (red blood cells).
- The odor of feces is caused by the chemicals indole, skatole, hydrogen sulfide and mercaptans, which are produced by bacterial action.

Many diseases and disorders can affect bowel function and produce abnormalities in the feces. Bleeding in the stomach or intestines may result in the passage of blood with the stool, which appears dark red, tarry or black. Fatty or greasy stool usually indicates pancreatic or small intestine afflictions.

One of the most common stool tests, the **fecal occult blood test** can be used to diagnose many conditions that cause bleeding in the gastrointestinal system including colorectal cancer or stomach cancer. A **fecal pH test** may be used to determine lactose intolerance or the presence of an infection. Steatorrhea can be diagnosed using a **fecal fat test** that checks for the malabsorption of fat.

FECAL FAT TEST

Dietary fat (primarily triglycerides) is digested in the duodenum by enzymes, such as pancreatic lipase, into smaller molecules of 1,2-diacylglycerols and free fatty acids, which can be absorbed through the wall of the jejunum of the small intestine and enter the circulation for metabolism and storage. As fat is a valuable nutrient, human feces normally contains very little of undigested fat.

In a normal person, absorption of fat is based on a variety of factors

- Bile production in the gallbladder or liver (if gallbladder has been removed).
- Production of digestive enzymes in pancreas.
- Normal functioning of intestines.

The fecal fat test is a diagnostic test for fat malabsorption conditions, which lead to excess fat in the feces (steatorrhea). A number of diseases of the pancreas and gastrointestinal tract are characterized by fat malabsorption. These include:

- Diseases affecting the intestines, such as:
  - Infections, including parasitic, bacterial or viral
  - Celiac disease, i.e. damage to the intestinal lining, caused by an allergy to gluten.
  - Inflammatory bowel disease (Crohn's disease, ulcerative colitis)
  - Pancreatic insufficiency, caused by:
    - Chronic pancreatitis (inflammation of the pancreas)
    - Pancreatic cancer
    - Cystic fibrosis, a genetic disease resulting in thick mucus secretions in the lungs and digestive tract.
- Diseases and conditions of the bile ducts and/or gallbladder
- Cancer: Tumor in pancreas or biliary duct

- Narrowing or blockage of the common bile duct, the main tube that carries bile from the liver and gallbladder to the intestines

People who have decreased absorption of fat often notice changes in their bowel habits. This is because the fat that is not digested is excreted in excess quantity in the feces. Stool is loose in consistency, almost like diarrhea. Stool with a high fat content also emits a foul odor and is likely to float.

Preparation of the Patient

For a 72-hour stool collection, follow the given instructions, which include consumption of nearly 100 grams (50–150 grams) of fat a day, for 2–3 days prior to and during the stool collection period.

Collection of the Sample

Collect stool (feces) sample in a clean container. Collect the stool each time one has a bowel movement during the 24-hour testing period. Label the container with name and age of the patient, and date. It should be delivered to the lab as soon as possible. If it is not fresh, bacteria in it will multiply and the test result may not be accurate.

For Children

Line the diaper of babies and toddlers with plastic wrap to carry out fecal fat testing. Try to place plastic in the back portion of the diaper, to prevent mixing of feces and urine.

Procedure

The container is thoroughly mixed to homogenize the feces, without using specific mixer equipment. A small sample from the feces is collected. The fat content is extracted with solvents and measured by saponification, i.e. turning the fat into soap by its reaction with alkali.

Results

Normal range for fecal fat is 7 grams over a 24-hour period. In patients with diarrhea, up to 12 grams of fat may be observed, since presence of diarrhea interferes with fat absorption, even when the diarrhea is not due to fat malabsorption.

FECAL OCCULT BLOOD

Fecal occult blood refers to the small amounts of blood in the stool that are too small to be seen or change the appearance of stool, meaning thereby that the frank blood is not visible to the naked eye in the given stool sample. Accordingly, the **fecal occult blood test** is used **to find blood in the feces**, or **stool**.

Blood in the stool may be a sign of colorectal cancer or other problems, such as ulcers or polyps. These are growths that develop on the inner wall of the colon and rectum.

Fecal Occult Blood Test

The fecal occult blood test is a method for detecting gastrointestinal bleeding, which may be the first sign of carcinoma of the colon or rectum. Although the false-positive rate for this test is low, the false-negative rate is high. The test also is more likely to detect lesions in the right (ascending) colon because these lesions bleed more than those in the left (descending) colon. Routine surveillance for colorectal cancer depends on periodic fecal occult blood testing combined with direct visualization of the lower colon with a sigmoidoscope.

The fecal occult blood test is a lab test used to check stool (feces) samples for hidden blood or blood products, that may arise due to small amount of bleeding in the gastrointestinal tract. Fecal occult blood is blood in feces that cannot be seen in stool or on toilet paper by yourself.

Occult blood in the stool indicates gastrointestinal bleeding that may be because of helminth infestation (hookworm infection). Other conditions associated with internal gastrointestinal bleeding are:

- Oesophagitis (inflammation of oesophagus; often associated with acid reflux)
- Esophageal cancer
- Gastritis
- Stomach ulcer or cancer
- Arteriovenous malformation
- Colon polyp
- Colon cancer
- Diverticulosis
- Ulcerative colitis
- Hemorrhoids

Test Methods

1. Chemically, using laboratory reagents, e.g. aminophenazone test.
2. Immunologically using ready-made reagents in kit tests (more expensive and usually less stable than aminophenazone test).

Procedure (Chemical Method)

1. Dispense about 7 mL of distilled water into a wide bore test tube.

2. Add a sample of feces, about 10–15 mm in diameter (taken from various parts of the specimen). Using a glass or plastic rod, emulsify the feces in the water.
3. Allow the fecal particle to settle or centrifuge the emulsified specimen.
4. Take three clean tubes and label them as **T** (patient's test), **N** (negative control) and **P** (positive control).
5. Add 5 mL of supernatant fluid from emulsified feces to **T**, 5 mL distilled water to **N** and 5 mL distilled water in which about 50 μL of whole blood has been mixed to **P.**
6. Layer 5 mL of working aminophenazone reagent on top of the fluid in each tube, i.e. pipette down the side of each tube. Do not mix.
7. Add 10 drops of the 10% hydrogen peroxide solution to each tube. Do not mix. Allow to stand for 1 minute.
8. Look for the appearance of a blue color where the aminophenazone reagent meets the sample or control solutions.

Report the Test Results

1. No color change: Negative test
2. Pale blue: Positive +
3. Dark blue: Positive + +
4. Blue-black: Positive + + +

Control

- Negative control: This should show no color change.
- Positive control: This should show a positive reaction.

Interpretation

- **A positive result** means that the test has detected blood.
- **A false positive** reaction may occur if the feces contains peroxidase-like substances.

PRACTICAL EXERCISES

1. Estimation of fecal fat
2. Test for occult blood

Important Question

1. Write notes on:
   i. Occult blood
   ii. Fecal fat

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